# MULTIGENE PHYLOGENY OF *EPICHLOË* SPECIES, FUNGAL SYMBIONTS OF GRASSES[1]

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## Abstract

*Epichloë* species are fungal symbionts (endophytes) of grasses, many of which are benign or mutualistic and have a balance of horizontal (contagious) and vertical (seed-borne) transmission, whereas others mainly transmit horizontally and are more antagonistic. Over the past eight years several *Epichloë* species have been described based largely on the biological species concept. We conducted a multi-gene phylogenetic analysis to evaluate these endophytes as phylogenetic species, and thereby assess the relationship of phylogenetic and biological species. Variation mainly in introns of genes encoding β-tubulin (*tub2*), translation elongation factor 1-α (*tef1*), and actin (*act1*) provided robust phylogenetic signal distinguishing the described *Epichloë* species. Outgroup rooting split the genus into two major groups. One group included most species with balanced transmission strategy, and in this group the phylogenetic and biological species concepts corresponded well. In contrast, these species concepts poorly corresponded for the other group, the *Epichloë typhina* complex, with predominantly antagonistic, horizontally transmitted endophytes. We suggest that the balance of vertical and horizontal transmission may promote ecological (host) specialization and subsequent genetic isolation as mechanisms promoting speciation; whereas strict horizontal transmission may select for broader host ranges, slow the development of genetically isolated species, and thereby increase lineage sorting effects that cause conflicts between phylogenetic and biological species.

*Key words:* biological species, Clavicipitaceae, *Epichloe*, grass endophytes, molecular phylogenetics, phylogenetic species, Poaceae, symbiosis.

Fungi in the family Clavicipitaceae (Ascomycota) are a diverse group of organisms capable of interacting with an even broader assemblage of hosts. Contained within the Clavicipitaceae are fungal and invertebrate pathogens (tribe Cordycipeae), and plant pathogens (tribes Ustilaginoideae, Clavicipeae, and Balansieae) (Diehl, 1950). Members of the tropical tribe Ustilaginoideae are biotrophs of bamboos, forming hardened sclerotic stromata on stems (Diehl, 1950), while the Clavicipeae destructively infect grass florets and replace host ovules with fungal sclerotia (Tudzynski et al., 1995). Fungi in the tribe Balansieae form perennial infections of grasses, yet cause little or no symptoms of disease. The extent of host colonization by members of this tribe varies from fairly localized, epibiotic infections to a more systemic, endophytic growth habit (Glenn et al., 1996). Species in the genus *Epichloë* lie at this endophytic extreme, systemically infecting cool-season grasses in the subfamily Pooideae (Schardl & Phillips, 1997). All *Epichloë* species are intercellular inhabitants of their host grasses, causing no visible symptoms of infection for the majority of the symbiont and host life cycles (Fig. 1). The only macroscopic sign of infection occurs as the grass reaches reproductive maturity and initiates flowering. At this stage, the endophytes may cause abortion of some or all inflorescences (grass-choke disease), and replace them with their own sexual structures (stromata). When a stroma is fertilized by spermatia from a stroma of an opposite mating type, the sexual state develops and culminates in production and ejection of contagious spores. The level of antagonism to the host is directly related to the level of fungal sexual expression, as a "strong choker" effectively shuts down host seed production (Schardl et al., 1997). While some *Epichloë* species seem to choke (stromatize) every developing host inflorescence, other species reproduce sexually on only a portion of the host tillers, the other inflorescences develop normally, and the endophyte enters into the developing ovule of the host seed. The result is vertical (asexual) transmission of the endophyte from the maternal host to her offspring.

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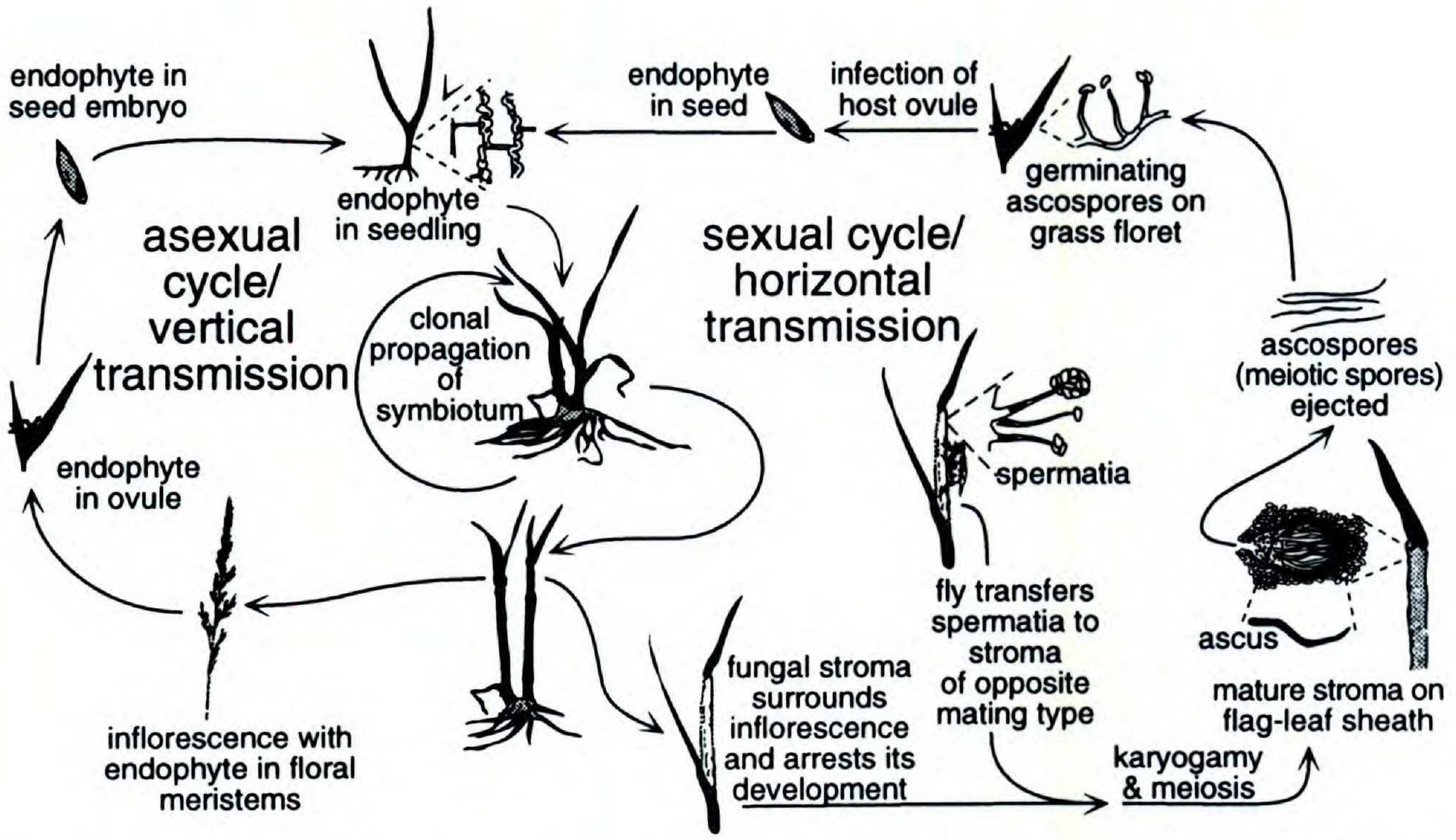


Figure 1. Generalized life cycle of *Epichloë* species using *E. festucae* in *Festuca rubra* as an example. Both transmission life cycles are exhibited by several *Epichloë* species (see text). Some *Epichloë* species (such as *E. typhina*) exhibit little or no asexual transmission in host seeds and are, therefore, predominantly transmitted horizontally via ascospores. Asexual derivatives of *Epichloë* species, conventionally classified in genus *Neotyphodium*, rely entirely on vertical transmission for their dissemination. Modified from Schardl et al. (1997).

Previous research based mainly on artificial mating tests has capitalized on the presence of the *Epichloë* sexual cycle and heterothallism (obligate outcrossing; White & Bultman, 1987) to circumscribe interfertility groups (mating populations) that correspond to, or approximate, biological species (Leuchtmann et al., 1994). Here we use, as a working definition of biological species, populations whose members have the potential to interbreed in nature to produce viable offspring, but who cannot interbreed with members of other species (Claridge et al., 1997; Mayr, 1940). To date, nine mating populations of *Epichloë* have been identified (Schardl et al., 1997).

Many grass endophytes appear to be restricted to an asexual lifestyle, and are only transmitted vertically (Sampson, 1933). In fact, the presence of such endophytes was widely recognized only after the realization that forage grasses infected with these asexual species were toxic to livestock such as cattle and sheep (Bacon et al., 1977). Subsequent studies have shown these endophytes to be responsible for producing novel secondary metabolites responsible for the toxicoses (Kennedy & Bush, 1983), as well as conferring on their hosts increased drought tolerance (Arechavaleta et al., 1989) and many other dramatic fitness enhancements (Schardl & Phillips, 1997). Despite the apparent inability of these asexual endophytes to spread contagiously, they can be abundant because they are seed transmitted at nearly 100% efficiency (Siegel et al., 1984). Although their hyphal morphology, conidiophore size and structure, and host associations all indicate their relatedness to *Epichloë* species, their lack of a sexual phase has precluded their classification based on a biological species concept.

The development of modern molecular techniques such as polymerase chain reaction (PCR) and DNA sequencing allows classification based on genetic relationships (phylogenies) to be considered. The strength of using gene sequence data for inferring relationships arises from a wealth of precisely comparable characters (aligned positions of nucleotide sequences) between even the most distantly related species (Felsenstein, 1988). Relationships emerging from such analyses have, in many cases, corroborated classification schemes based on morphological characteristics (Hillis, 1987). Molecular data have also been used to either strengthen or weaken support for hypotheses regarding organismal and cellular evolution (Hillis, 1987). For example, gene sequence data indicate that cellular organelles such as mitochondria and chloroplasts are most closely related to some free-living bacterial species, lending credence to the endosymbiont theory of the modern-day eukaryote cell as a composite of two or more once distinct prokaryote species (Margulis, 1991).

Gene sequence data also allows phylogenetic

inferences for organisms that lack distinguishing features for reliable morphological classification. Because of rather little morphological variation many sexual endophytes were originally classified as *Epichloë typhina.* Recently, a number of species have been described based on molecular phylogenetic evidence, interfertility groups, and morphology (Leuchtmann & Schardl, 1998; Leuchtmann et al., 1994; Schardl & Leuchtmann, 1999; White, 1993, 1994). A phylogeny has been inferred from partial sequences of 26S large subunit rRNA genes from *Epichloë* species and other members of the family Clavicipitaceae (Kuldau et al., 1997). Those endophytes assigned to the genus *Epichloë* through morphological data and mating tests all formed a monophyletic clade within the Clavicipitaceae. A more comprehensive analysis within the genus *Epichloë* was conducted using non-coding regions of the β-tubulin gene (*tub2*) (Schardl et al., 1997). Each sexual isolate contained a single *tub2* copy, reflecting the haploid state of the *Epichloë* mycelium. Most but not all mating populations correspond to well defined clades in phylogenetic analyses based mainly on *tub2* introns. However, more detailed analyses are needed to further test the inferred relationships.

Similar analysis of *tub2* sequence data obtained from the strictly asexual grass endophytes revealed quite novel findings. The majority of these isolates actually appeared to have multiple *tub2* copies (Schardl et al., 1994; Tsai et al., 1994; Moon et al., 2000; K.D.C. & C.L.S., unpublished obs.). Through a combination of restriction endonuclease digestion and selective priming in PCR, as well as by cloning, these copies could be isolated and sequenced independently. Inclusion of these sequences in a phylogenetic analysis of *Epichloë* species revealed that each *tub2* copy contained in the genome of an asexual grass endophyte aligned most closely with that of a different sexual *Epichloë* species (Schardl et al., 1994; Tsai et al., 1994). For example, *Neotyphodium coenophialum,* a common asexual endophyte of tall fescue [*Lolium arundinaceum* (Schreb.) S. J. Darbyshire = *Festuca arundinacea* Schreb.] was found to harbor three *tub2* copies corresponding with those of three different *Epichloë* species: *E. festucae, E. typhina,* and *E. baconii* (Tsai et al., 1994). Similar results were obtained by analysis of other genes (specifically, those utilized in this paper; K.D.C. & C.L.S., unpublished data). Furthermore, other asexual endophytes from *Festuca* L. spp., *Lolium* L. spp., *Poa* L. spp., and *Stipa* L. spp. have genes derived from different combinations of *Epichloë* species, such as *E. baconii* with *E. typhina* (Tsai et al., 1994), *E. baconii* with *E. bromicola* (Moon et al., 2000), *E. amarillans* with *E. typhina* (Schardl & Wilkinson, 2000), *E. festucae* with *E. typhina* (K.D.C., unpublished), *E. festucae* with *E. elymi, E. bromicola* with *E. typhina,* and *E. bromicola* with *E. festucae* (K.D.C., unpublished). Based on these findings, most asexual endophytes related to *Epichloë* spp. are likely to be genetic hybrids arising through the parasexual process of hyphal anastomosis (Schardl et al., 1994; Schardl & Wilkinson, 2000), and the asexual tall fescue endophyte is apparently derived from at least two such hybridizations. The genus *Neotyphodium* has been adopted to contain such endophytes with close relationships to *Epichloë* spp., but lacking a sexual stage in their life cycles (Glenn et al., 1996). A morphological difference that often distinguishes *Neotyphodium* spp. from their *Epichloë* relatives is that the asexual endophytes tend to have larger conidia, probably as a result of increased genome content due to hybrid origins (Kuldau et al., 1999).

A potential problem that arises when sequence data are used to construct phylogenies is whether or not the gene tree accurately represents the true evolutionary relationships between species (species tree). For example, similarities between two sequences can arise from convergence, gene conversion, or homology (common ancestry). Homology can further be divided into orthology (common ancestry tracing back to a speciation event) and paralogy (common ancestry tracing back to a gene duplication event) (Moritz & Hillis, 1996). Sampling of paralogous sequences can cause incorrect inferences about species phylogeny, so only orthologous sequences should be evaluated. Therefore, the genes chosen in such analyses are of paramount importance if one wishes to obtain an accurate evolutionary picture.

Another way of measuring the accuracy of a gene tree in reflecting the actual species tree is to sample other genes. If trees developed from different, independently assorting genes contain identical branching orders and taxa placement, this can be regarded as strong support for these estimations as accurately revealing the evolutionary relationships among the species being studied. An additional advantage to multi-gene phylogenetics is that populations that are, or have been, interbreeding can be distinguished from those that are genetically isolated in nature (Geiser et al., 1998; Burt et al., 1996). This is important for fungal evolutionary biology because on the one hand many lack a known sexual stage, and on the other hand the ability to mate in experimental crosses (biological species) does not guarantee that the process is relevant to natural populations. This paper reports the char-

acterization and analysis of two more genes in the *Epichloë* genome. Portions of the translation elongation factor 1-α (*tef1*) gene (O'Donnell et al., 1998) and the actin (*act1*) gene (Fidel et al., 1988), with variable introns, were amplified and sequenced for this group of closely related grass endophytes. The sequence data were used to construct separate gene phylogenies, which could be compared to those obtained from *tub2* sequences. The results indicate different phylogenetic patterns for symbiont species that themselves differ in relative mutualistic or antagonistic character.

## Materials and Methods

### Biological materials

Fungal isolates, their hosts, and sequence accession numbers are listed in Table 1. Endophytes were isolated by plating infected, surface-sterilized leaf sheath and stem material onto potato dextrose agar (PDA, Difco Laboratories, Detroit, Michigan) containing streptomycin and penicillin (1 mg $L^{-1}$ each). Plates were checked regularly for contamination and endophytic growth. Most strains grew out 5–14 days after plating. Isolates were stored in 50% glycerol at −80°C (Schardl & An, 1993) and on PDA slant cultures overlain with autoclaved light mineral oil.

### Mating tests

Plants naturally infected with *Epichloë* were vernalized over winter at an experimental garden in Zurich, Switzerland, to induce stromata formation. The following spring, plants were moved into a greenhouse where mating tests were performed on emergent stromata. Spermatia were transferred between mating types by rubbing stromata of opposite mating type together, or by applying cultures containing conidia onto stromata. Successful matings between opposite mating types (of the same mating population) were evidenced by the production of tan to orange perithecia after 3–6 weeks, from which ascospores could be collected. Single ascospore isolates were grown on media and subjected to isozyme analysis. Independent assortment of parental isozyme alleles in ascospore progeny confirmed successful matings.

### Preparation of genomic DNA

Subcultures were generated by grinding a small amount of fungal mycelium in sterile water using an autoclaved mini-pestle and spreading approximately 300 μl of this suspension atop sterile cellophane disks on PDA plates. The permeable cellophane allowed hyphal growth and facilitated subsequent harvest of mycelium. Cultures were incubated at room temperature for two weeks, after which mycelium was harvested and freeze dried. Total fungal DNA was prepared as previously described (Byrd et al., 1990; Schardl et al., 1997) or, preferably, the method of Al Samarrai and Schmid (2000).

### Assessment of gene linkage

To assess the possibility of linkage between the three genes used in this analysis, spermatia from isolate ATCC200736 (*Epichloë typhina* from *Lolium perenne*) were used to fertilize stromata of isolate ATCC200740 (*E. typhina* from *Dactylis glomerata*). Since these two *E. typhina* isolates are of opposite mating type, perithecia developed from which ascospores were obtained. Inheritance of parental alleles in each progeny was assessed by polymerase chain reaction (PCR) with primers specific for each *tub2*, *tef1*, and *act1* allele. All primers are listed in Table 2. Amplification of *tub2* spanning introns 1–3 was performed using non-selective primer tub2-exon1d–1 with selective primers tub2-intron3u–200736 (selective for *E. typhina* ATCC200736) and tub2-intron3u–200740 (selective for *E. typhina* ATCC200740). The non-selective primer annealed to the exon 1–intron 1 junction while both selective primers annealed to complementary regions within intron 3. A region of the *act1* gene approximately 300 bp long (including introns 2–4) was amplified using two selective primers for each parental isolate. Primers act1-intron2d–200736 and act1-intron-4u-200736, respectively, annealed to sites within introns 2 and 4 of the ATCC200736 *act1* allele. Similarly, primers act1-intron2d–200740 and act1-intron4u–200740 annealed to these same regions of the ATCC200740 *act1* allele. Partial amplification of the *tef1* gene, including introns 1–2, was performed using the non-specific primer tef1-exon1d with specific primers tef1-intron3u–200736 for ATCC200736 or tef1-intron3u–200740 for ATCC200740. The non-specific primer anneals to conserved sequence within exon 1, while both isolate specific primers anneal to complementary sequence within intron 4. PCR was performed in 50 μl reactions containing 0.2 μM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP; Boehringer Mannheim, Indianapolis, Indiana), 1 X PCR buffer (10 mM tris(hydroxymethyl)aminomethane hydrochloride [Tris-HCl] pH 8.3, 15 mM $MgCl_2$), 500 nM of each primer (see below), 10 ng total fungal DNA, and 0.02 U $\mu l^{-1}$ of AmpliTaq gold DNA polymerase (Perkin-Elmer, Foster City, California). The thermal cycler (Perkin-Elmer model 2400) was programmed as fol-

Table 1. *Epichloë* species used in this study. All isolates are housed in permanent reference storage at the American Type Culture Collection (ATCC) except those designated with E prefixes, which are kept in cold storage at the University of Kentucky. nd = not determined.

| Fungal species | Isolate | Host species | GenBank accession number | | |
|---|---|---|---|---|---|
| | | | *act1* | *tub2* | *tef1* |
| *Claviceps purpurea* (Fr.) Tul. | ATCC20102 | *Secale cereale* L. | AF276509 | AF062646 | AF276508 |
| *Echinodothis tuberiformis* (B. & Rav.) Atk. | ATCC201937 | *Arundinaria tecta* (Walter) Muhl. | AF276511 | L78268 | AF276510 |
| *Epichloë amarillans* J. F. White | ATCC200743 | *Sphenopholis obtusata* (Michx.) Scribn. | AF240082 | L06958 | AF231191 |
| *E. amarillans* | ATCC201670 | *Sphenopholis obtusata* | nd | AF062426 | nd |
| *E. amarillans* | ATCC200744 | *Agrostis hiemalis* (Walter) Britton et al. | AF240083 | L06959 | AF231192 |
| *E. amarillans* | ATCC90815 | *Agrostis hiemalis* | nd | AF250732 | nd |
| *E. baconii* J. F. White | ATCC200745 | *Calamagrostis villosa* (Chaix) Gmelin | AF240086 | L78270 | AF231196 |
| *E. baconii* | ATCC76552 | *Agrostis stolonifera* L. | AF240084 | L06961 | AF231193 |
| *E. baconii* | ATCC90167 | *Agrostis tenuis* Sibth. | AF240085 | L78279 | AF231194 |
| *E. baconii* | ATCC200746 | *Agrostis tenuis* | nd | AF250733 | AF231195 |
| *E. brachyelytri* Schardl & Leuchtmann | ATCC200752 | *Brachyletrum erectum* (Schreb.) Beauv. | AF240087 | L78271 | AF231197 |
| *E. brachyelytri* | ATCC200753 | *Brachyletrum erectum* | nd | AF250734 | AF231198 |
| *E. brachyelytri* | ATCC200754 | *Brachyletrum erectum* | nd | AF250735 | AF231199 |
| *E. brachyelytri* | ATCC201561 | *Brachyletrum erectum* | AF240088 | AF062427 | AF231200 |
| *E. brachyelytri* | ATCC201560 | *Brachyletrum erectum* | nd | AF250736 | AF231201 |
| *E. bromicola* Leuchtmann & Schardl | ATCC200749 | *Bromus erectus* Huds. | AF240089 | L78289 | AF231202 |
| *E. bromicola* | ATCC200750 | *Bromus erectus* | AF240090 | L78290 | AF231203 |
| *E. bromicola* | ATCC201558 | *Bromus ramosus* Huds. | AF240091 | AF062430 | AF231204 |
| *E. bromicola* | ATCC201559 | *Bromus benekenii* (Lange) Trimen | AF240092 | AF250737 | AF231205 |
| *E. clarkii* J. F. White | ATCC200742 | *Holcus lanatus* L. | AF240093 | L78281 | AF231206 |
| *E. clarkii* | ATCC200741 | *Holcus lanatus* | nd | AF250738 | AF231207 |
| *E. clarkii* | E1155 | *Holcus lanatus* | nd | AF250739 | nd |
| *E. clarkii* | E426 | *Holcus lanatus* | nd | AF250740 | nd |
| *E. clarkii* | ATCC90168 | *Holcus lanatus* | nd | AF250741 | nd |
| *E. elymi* Schardl & Leuchtmann | ATCC200850 | *Elymus virginicus* L. | AF240094 | L78273 | AF231208 |
| *E. elymi* | ATCC201551 | *Elymus canadensis* L. | AF240095 | L06962 | AF231209 |
| *E. elymi* | ATCC201553 | *Elymus virginicus* | nd | AF062428 | nd |
| *E. elymi* | ATCC201554 | *Elymus virginicus* | AF240096 | AF250742 | nd |
| *E. elymi* | ATCC201555 | *Elymus villosus* Muhl. ex Willd. | nd | AF250743 | nd |
| *E. elymi* | ATCC201556 | *Elymus hystrix* L. | AF240096 | AF250744 | nd |
| *E. elymi* | ATCC201557 | *Elymus hystrix* | nd | AF250745 | nd |
| *E. festucae* Leuchtmann, Schardl, & Siegel | ATCC90661 | *Festuca rubra* subsp. *rubra* (Gaud.) Hayek | AF240098 | L06955 | AF231210 |
| *E. festucae* | ATCC201550 | *Festuca rubra* subsp. *commutata* Gaudin | AF240100 | L06957 | AF231211 |

Table 1. Continued.

| Fungal species | Isolate | Host species | GenBank accession number | | |
|---|---|---|---|---|---|
| | | | *act1* | *tub2* | *tef1* |
| *E. festucae* | ATCC90660 | *Festuca rubra* subsp. *commutata* | nd | AF250746 | AF231214 |
| *E. festucae* | E028 | *Festuca longifolia* Thuill. | AF240099 | L06956 | AF231213 |
| *E. festucae* | E434 | *Lolium giganteum* (L.) S. J. Darbyshire | nd | L78286 | AF231212 |
| *E.* cf. *festucae* | E1157 | *Koeleria cristata* Pers. | nd | AF250747 | AF231215 |
| *E. glyceriae* Schardl & Leuchtmann | ATCC200747 | *Glyceria striata* (Lam.) Hitchc. | AF240101 | L78275 | AF231216 |
| *E. glyceriae* | ATCC200755 | *Glyceria striata* | AF240102 | L78276 | AF231217 |
| *E. sylvatica* Leuchtmann & Schardl | ATCC200748 | *Brachypodium sylvaticum* (Huds.) P. B. | AF240104 | L78278 | AF231218 |
| *E. sylvatica* | ATCC200751 | *Brachypodium sylvaticum* | AF240103 | L78291 | AF231219 |
| *E. typhina* (Pers.:Fr.) Tul. | ATCC200736 | *Lolium perenne* L. | AF240105 | X52616 | AF231220 |
| *E. typhina* | E432 | *Lolium perenne* | AF240106 | AF250752 | AF231221 |
| *E. typhina* | ATCC200738 | *Anthoxanthum odoratum* L. | AF240107 | L78288 | AF231222 |
| *E. typhina* | ATCC200737 | *Anthoxanthum odoratum* | nd | AF250752 | nd |
| *E. typhina* | ATCC200739 | *Brachypodium pinnatum* (L.) P. Beauv. | AF240108 | L78292 | AF231223 |
| *E. typhina* | E1016 | *Brachypodium pinnatum* | AF240109 | AF250749 | AF231224 |
| *E. typhina* | ATCC200740 | *Dactylis glomerata* L. | AF240110 | L78274 | AF231225 |
| *E. typhina* | ATCC200849 | *Dactylis glomerata* | nd | L78287 | nd |
| *E. typhina* | ATCC200851 | *Phleum pratense* L. | AF240111 | L78280 | AF231226 |
| *E. typhina* | E348 | *Phleum pratense* | AF240112 | L78277 | AF231227 |
| *E. typhina* | ATCC201667 | *Poa nemoralis* L. | AF240114 | AF062429 | AF231229 |
| *E. typhina* | ATCC201668 | *Poa nemoralis* | AF240115 | AF250756 | AF231230 |
| *E. typhina* | E1015 | *Poa trivialis* L. | nd | AF250748 | nd |
| *E. typhina* | E1153 | *Poa trivialis* | nd | AF250751 | nd |
| *E. typhina* | E428 | *Poa trivialis* | nd | AF250755 | nd |
| *E. typhina* | E1158 | *Arrhenatherum elatius* (L.) P. Beauv. | nd | AF250753 | nd |
| *E. typhina* | ATCC201666 | *Poa silvicola* Guss. | AF240113 | L78285 | AF231228 |
| *E. typhina* | E1019 | *Poa silvicola* | nd | AF250750 | nd |
| *E. typhina* | ATCC201669 | *Poa pratensis* L. | AF240116 | L78284 | AF231231 |
| *E. typhina* | E1154 | *Poa pratensis* | nd | AF250757 | nd |
| *E. typhina* | E1020 | *Poa pratensis* L. subsp. *angustifolia* (L.) Lej. | nd | nd | AF231232 |

Table 2. Oligonucleotide primers used in this study. Primers are listed in order of appearance in text.

| Oligonucleotide designation | Gene | Sequence (5′–3′) | Position | Orientation |
|---|---|---|---|---|
| tub2-exon1d-1 | *tub2* | GAGAAAATGCGTGAGATTGT | exon 1–intron 1 junction | downstream |
| tub2-intron3u-200736 | *tub2* | GCATGATAACACTTTTCCTTTCCA | intron 3 | upstream |
| tub2-intron3u-200740 | *tub2* | GCATGATAACACGTTTTATTTGCT | intron 3 | upstream |
| act1-intron2d-200736 | *act1* | GCGTAGACCCACGCAACGTA | intron 2 | downstream |
| act1-intron4u-200736 | *act1* | GTGCATGTTTGCCAGCCAGA | intron 4 | upstream |
| act1-intron2d-200740 | *act1* | GCATAGACCCACGCAACGTG | intron 2 | downstream |
| act1-intron4u-200740 | *act1* | GTGCATGTTTGCCAGCCAGT | intron 4 | upstream |
| tef1-exon1d | *tef1* | GGGTAAGGACGAAAAGACTCA | exon 1 | downstream |
| tef1-intron3u-200736 | *tef1* | ATCACGCGAATGTCATGTTG | intron 4 | upstream |
| tef1-intron3u-200740 | *tef1* | ATCACGCGAATGTCATGTTA | intron 4 | upstream |
| tub2-exon4u-1 | *tub2* | TGGTCAACCAGCTCAGCACC | exon 4 | upstream |
| tef1-exon6u-1 | *tef1* | CGGCAGCGATAATCAGGATAG | exon 6 | upstream |
| act1-exon1d-1 | *act1* | TAATCAGTCACATGGAGGGT | exon1–intron 1 junction | downstream |
| act1-exon6u-1 | *act1* | AACCACCGATCCAGACAGAGT | exon 6 | upstream |
| act1-exon3u-1 | *act1* | CTTGCACATGCCCGAACTACC | exon 3 | upstream |
| act1-exon3d-1 | *act1* | GGTAGTTCGGGCATGTGCAAG | exon 3 | downstream |

lows: one incubation at 94°C for 9 min.; then 40 cycles of 94°C for 60 sec., 60°C for 60 sec., and 72°C for 60 sec.

### SEQUENCING OF GENOMIC DNA SEGMENTS

Sequences of each gene were obtained directly from PCR-amplified products. All oligonucleotide primers used for PCR and sequencing are listed in Table 2 along with their corresponding positions and orientations in the genes. The 5′ portions of *tub2*, inclusive of introns IVS1, IVS2, and IVS3, were amplified using non-selective primers tub2-exon1d–1 and tub2-exon4u–1. A ca. 850 bp fragment of *tef1*, including the first five introns, was amplified using primers tef1-exon1d–1 and tef1-exon6u–1. A ca. 700 bp fragment of the *act1* gene, including introns 1–5, was amplified by PCR using primers act1-exon1d–1 and act1-exon6u–1. Internal regions of the PCR-amplified product were sequenced with primers act1-exon3u–1 and act1-exon3d–1. All amplification products were verified by agarose gel (0.8%) electrophoresis. Water blanks were included as controls to identify possible contamination. Successfully amplified products were purified for sequencing using Quiaquick spin columns (Quiagen, Chatsworth, California). PCR was performed as described above, except that the temperature program was as follows: one incubation at 94°C for 9 min.; then 40 cycles of 94°C for 60 sec., 55°C for 60 sec., and 72°C for 60 sec. Reaction mixtures were as described above.

PCR products were sequenced by the Sanger method (Thomas & Kocher, 1993) with a Perkin-Elmer GeneAmp PCR System 2400 using rhodamine-labeled dideoxynucleotide triphosphates or BigDye Terminator Cycle sequencing kit (Perkin-Elmer). Sequence reaction products were analyzed using an ABI model 310 capillary electrophoresis genetic analyzer (Perkin-Elmer). Both DNA strands were sequenced.

### PHYLOGENETIC ANALYSIS

Sequences were aligned using the PILEUP program in the Wisconsin Package (GCG, 1996). PILEUP parameters were adjusted empirically; a gap penalty of one and a gap extension penalty of zero resulted in accurate alignment of intron-exon junctions and in a reasonable alignment of the introns within the ingroup (*Epichloë/Neotyphodium* spp.) for individual analysis of *tub2*, *act1*, and *tef1* genes, and for a combined data set using appended sequences of all three genes for representative isolates from each *Epichloë* species, as well as from outgroup taxa, *Claviceps purpurea* ATCC20102 and *Echinodothis tuberiformis* ATCC201937. Both outgroup taxa are in the family Clavicipitaceae and are among the closest known relatives of *Epichloë* (Glenn et al., 1996). No ambiguous alignments were evident for the ingroups, but slight realignment of outgroup sequences were needed and were performed by eye. Maximum parsimony (MP) employed the branch-and-bound option in PAUP* version 4.0 beta 4 (Swofford, 1998) for exact solutions. For parsimony analysis, character changes were unweighted and unordered; gaps were treated as missing information. Robustness of the data was estimated by bootstrap replications (Swofford et al., 1996). All branches receiving 70% or higher boot-

Table 3. Linkage analysis between *act1*, *tub2*, and *tef1-alpha* genes. Parental alleles represented by letters a (for *E. typhina* ATCC200736) and b (for *E. typhina* ATCC200740). Parental isolates were chosen based on differential polymorphism in each allele detectable by selective primers in PCR.

| Fungal isolate | *act1* | *tef1* | *tub2* | *act1/tef1* | *act1/tub2* | *tef1/tub2* |
|---|---|---|---|---|---|---|
| *E. typhina* parental isolates used in cross | | | | | | |
| ATCC200736 | a | a | a | aa | aa | aa |
| ATCC200740 | b | b | b | bb | bb | bb |
| Progeny isolates obtained | | | | | | |
| E386.003 | a | a | b | aa | ab | ab |
| E386.009 | a | b | a | ab | aa | ba |
| E386.026 | b | b | a | bb | ba | ba |
| E386.027 | b | b | b | bb | bb | bb |
| E386.042 | a | b | b | ab | ab | bb |
| E386.043 | a | b | b | ab | ab | bb |
| E386.045 | a | a | a | aa | aa | aa |
| E386.057 | a | b | b | ab | ab | bb |
| E386.084 | a | b | a | ab | aa | ba |
| E386.180 | a | b | a | ab | aa | ba |
| aa : bb : ab : ba | | | | 2:2:6:0 | 4:1:4:1 | 1:4:1:4 |

strap values were considered well supported. For distance-based analysis a number of different methods were employed to derive pairwise distances. These included Jukes-Cantor and Kimura two-parameter distances [transition/transversion (ts/tv) = 2], and various gamma values to adjust for between-site variation. Distance trees were inferred by neighbor-joining (NJ) implemented in PAUP* version 4.0 beta 4 (Swofford, 1998) with random taxon additions. All such distance trees were similar to the MP trees, and are therefore not shown. Maximum likelihood (ML) trees were constructed using PUZZLE in PAUP* version 4.0 beta 4. A Hasegawa-Kishino-Yano model (Swofford et al., 1996) was employed with transition/transversion = 2.0. PUZZLE uses quartet puzzling to create trees for all possible quartets of taxa via maximum likelihood. These quartet trees serve as starting points to reconstruct a set of optimal trees for all taxa. The majority-rule consensus of these trees defines the quartet puzzling tree and shows well-supported branches (Strimmer & von Haeseler, 1996). Partition homogeneity analysis was performed in PAUP* version 4.0 beta 4 on each two-gene combination in the combined sequence alignment.

## Results

### Linkage analysis

To check if the three genes selected for phylogenetic analysis—namely, *tub2*, *act1*, and *tef1*—were unlinked, Mendelian segregation was assessed for variant alleles in an *Epichloë typhina* cross. Ten progeny were analyzed for alleles derived from the parents, ATCC200736 (male) and ATCC 200740 (female). The occurrence of recombinant progeny was assessed for each gene pair. Ratios of parental to recombinant progeny genotypes indicated no close linkage between any of these genes in *E. typhina* (Table 3).

### Mating tests

Past mating tests indicated that the currently described *Epichloë* species mostly correspond to interfertile groups (Leuchtmann & Schardl, 1998; Schardl & Leuchtmann, 1999). Exceptions were some instances of nonreciprocal interfertility among *E. typhina* isolates, and high fertility between *E. clarkii* and *E. typhina*. Several additional mating tests were conducted to establish species affinities of new isolates and mating tests that had not previously been conducted for technical reasons. A new *Epichloë* isolate from *Arrhenatherum elatius* was fully compatible in matings with *E. typhina* stromata on *Dactylis glomerata*, as were the reciprocal matings. The ascospore progeny were viable and exhibited segregation of parental allozymes. An isolate from *Poa nemoralis* was compatible with at least some strains from *Brachypodium sylvaticum*, but the matings exhibited low fertility with few asci containing ascospores and a very low germination rate of the ascospores. These matings were also confirmed by allozyme analysis. *Epichloë typhina* stromata from *P. nemoralis* mated successfully with *Brachypodium pinnatum* isolates, although fertility

was intermediate (moderate ascospore germination rate). *Epichloë clarkii* isolates from *Holcus lanatus* successfully mated with *E. typhina* stromata on *Poa trivialis*. *Epichloë festucae* fertilized with a new isolate from *Koeleria cristata* reacted by forming protoperithecia, but no mature perithecia were observed and no ascospores were obtained. *Epichloë festucae* stromata on *Festuca rubra* mated successfully with endophyte isolates from *Lolium giganteum* (= *Festuca gigantea*), *Festuca valesiaca*, *F. glauca*, and *F. heterophylla*. Matings were attempted between stromata of *E. amarillans* isolates on *Sphenopholis obtusata* and spermatia of the *E. amarillans* ex-type (ATCC200744) from *Agrostis hiemalis*. Abundant perithecia gave rise to viable ascospores, but in the 10 ascospore isolates analyzed, only maternal allozyme alleles were observed.

### PHYLOGENETIC RELATIONSHIPS BASED ON *ACT1/TUB2/TEF1* COMBINED DATA

An MP tree (Fig. 2) was constructed from the combined data set of *act1*, *tub2*, and *tef1* intron sequences, and included representatives of all described *Epichloë* species and two clavicipitaceous outgroups, *Claviceps purpurea* and *Ech. tuberiformis*. The tree shown in Figure 2 reflects the overall diversity within clades and described *Epichloë* species. However, results described later indicated some incongruences between gene trees due to reticulation involving *E. typhina*. For this reason the positions of *E. typhina* isolates did not reflect their positions in all gene trees. To test that the combined analysis gave a valid result for relationships between species, it was rerun with only two representatives of *E. typhina*, and again without any *E. typhina* sequences (data not shown). In all cases the interrelationships of the remaining taxa were the same.

In bootstrap analysis (again using MP) of the combined data set most branches were strongly supported, but the placement of the root received only weak support (Fig. 3). This was not surprising because of the considerable sequence divergence between the ingroup and outgroup taxa, making unambiguous alignment difficult. Nevertheless, the inferred root was very close to the midpoint root of each individual gene tree (discussed later and shown in Figs. 4, 7, 11). Assuming its correct placement in the combined data analysis, the root split the *Epichloë* genus into two major groups (Fig. 3): One group, comprising *Epichloë amarillans*, *E. baconii*, *E. festucae*, *E. bromicola*, *E. elymi*, *E. glyceriae*, and *E. brachyelytri*, is henceforth designated

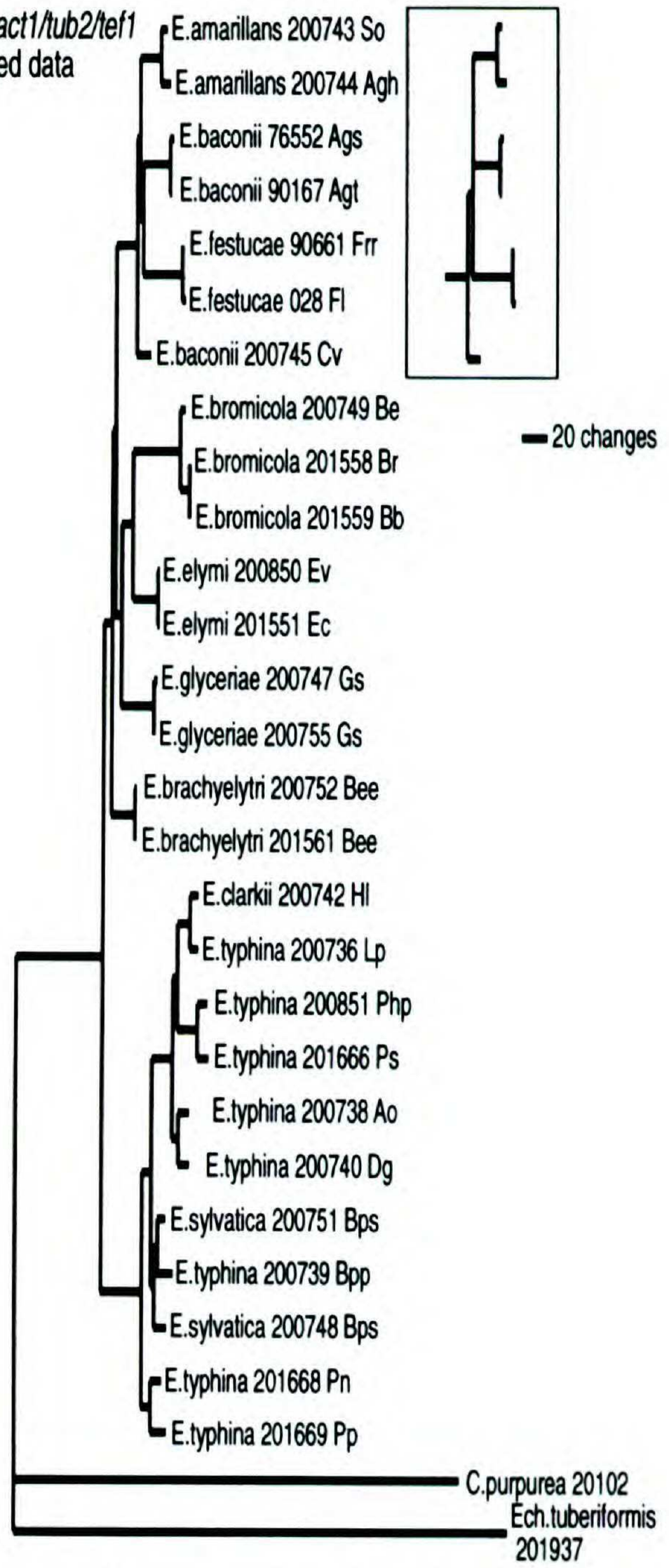


Figure 2. MP tree generated from the combined *act1/tub2/tef1* data set including representatives of each major *Epichloë* clade, *Claviceps purpurea* and *Echinodothis tuberiformis* (outgroups). Branch-and-bound search resulted in 2 MP trees, differing slightly in the *E. festucae/E. baconii/E. amarillans* clade. The alternative resolution of this clade is shown in the smaller box at right. The number of parsimony-informative characters = 379; tree length = 1350 steps; consistency index = 0.8622; retention index = 0.8502; rescaled consistency index = 0.7331; outgroup rooting with basal polytomy. Bar represents 20 inferred nucleotide substitutions. Letters following isolate number refer to abbreviated host designation. Full host names are listed in Table 1.

the "main group" of *Epichloë* species. The second group, henceforth called the "*E. typhina* complex," included *E. typhina* isolates from several host species, as well as *E. clarkii* (from *Holcus lanatus*) and *E. sylvatica* (from *Brachypodium sylvaticum*). A very strongly supported clade within the *E. typhina*

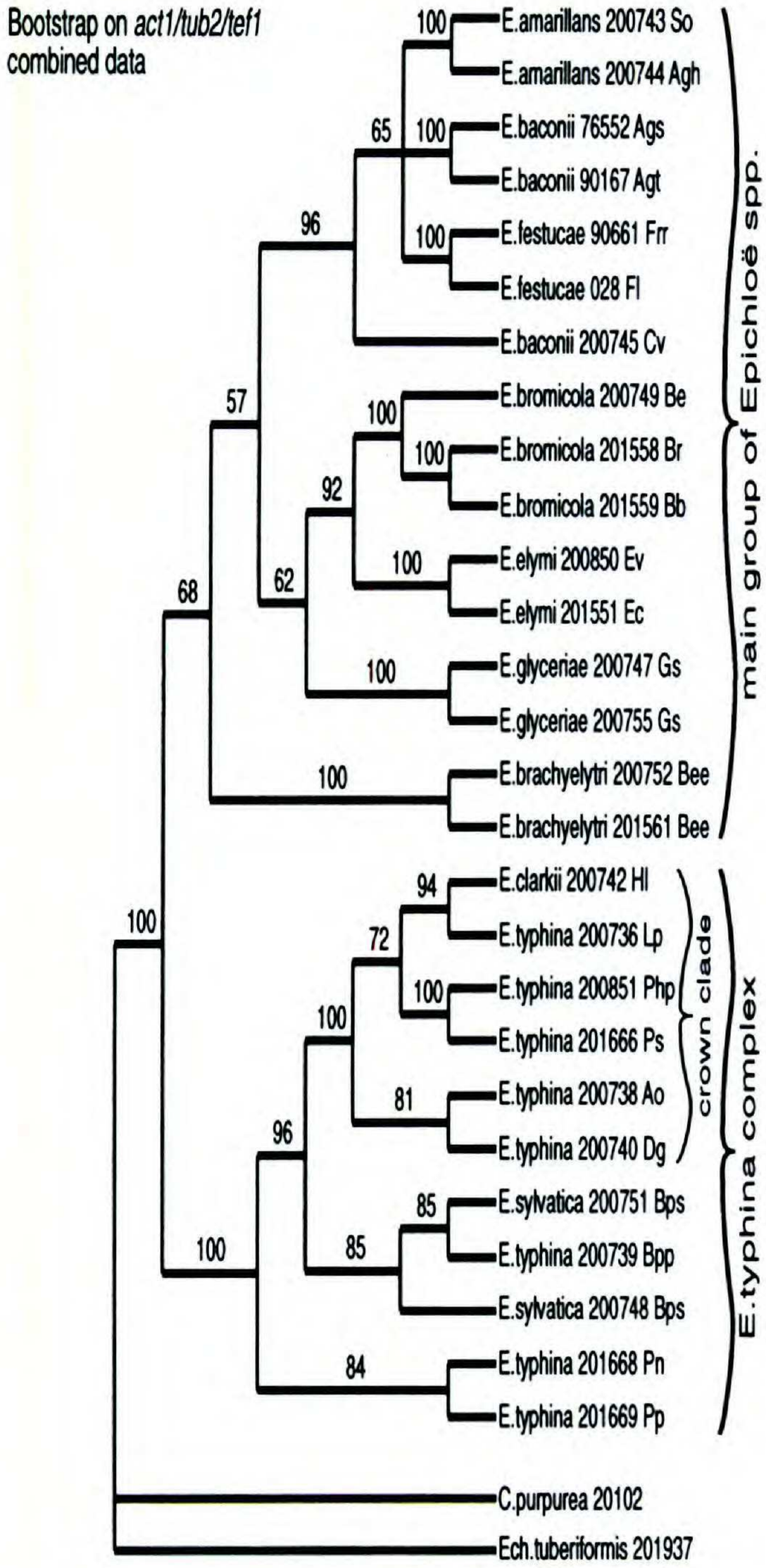


Figure 3. Bootstrap tree generated from the combined *act1/tub2/tef1* data set. Numbers at branches are the percentage of trees containing the corresponding clade based on 1000 bootstrap replications. Only bootstrap values greater than 70% are shown and considered supportive of the clades indicated by the branches.

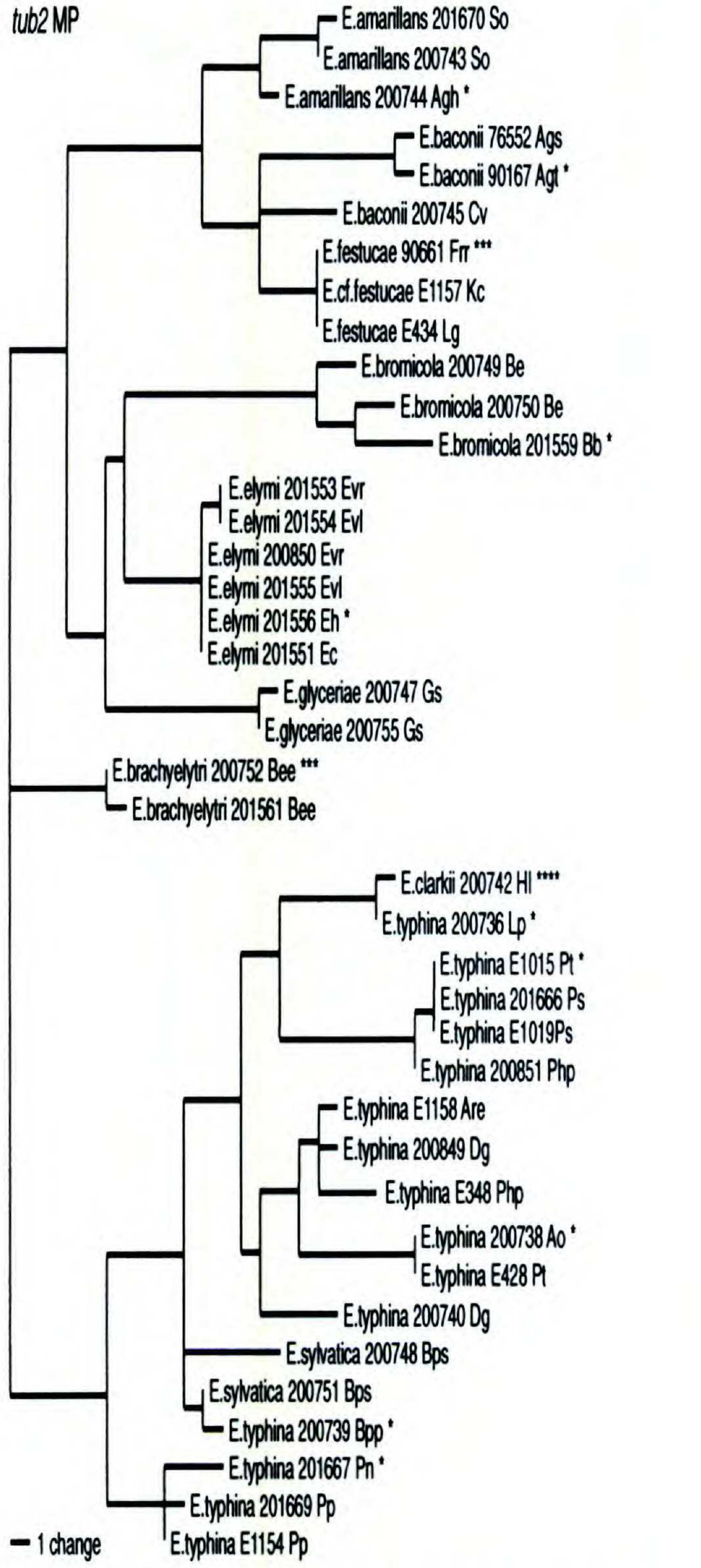


Figure 4. *Epichloë* spp. *tub2* gene tree based on maximum parsimony (MP) analysis of introns 1–3. Shown is the single MP tree obtained by branch-and-bound search. Number of parsimony-informative characters = 82; uninformative characters = 21; tree length = 143 steps; consistency index = 0.8182; retention index = 0.9426; rescaled consistency index = 0.7712; midpoint root is at the left edge. Bar represents one inferred nucleotide substitution. Asterisks (*) indicate the number of additional isolates (ranging from one to four), sampled from the same host genus, that had identical sequence.

complex was designated the crown clade (Fig. 3), and includes among others, *E. typhina* from *Dactylis glomerata* (which was among the original hosts on which Persoon described *E. typhina* and which was later selected as host of the lectotype) and *E. clarkii* from *Holcus lanatus*.

### β-TUBULIN GENE PHYLOGENY

The *tub2* data set included sequences from 59 isolates of *Epichloë* spp. Phylogenies derived by MP (Figs. 4, 5), NJ (not shown), and ML quartet puzzling (Fig. 6) were consistent with each other and with the *tub2* relationships previously reported among *Epichloë* species (Schardl & Leuchtmann, 1999). Both MP bootstrap analysis (Fig. 5) and ML quartet puzzling (Fig. 6) strongly supported the two major groups elucidated by the combined data set.

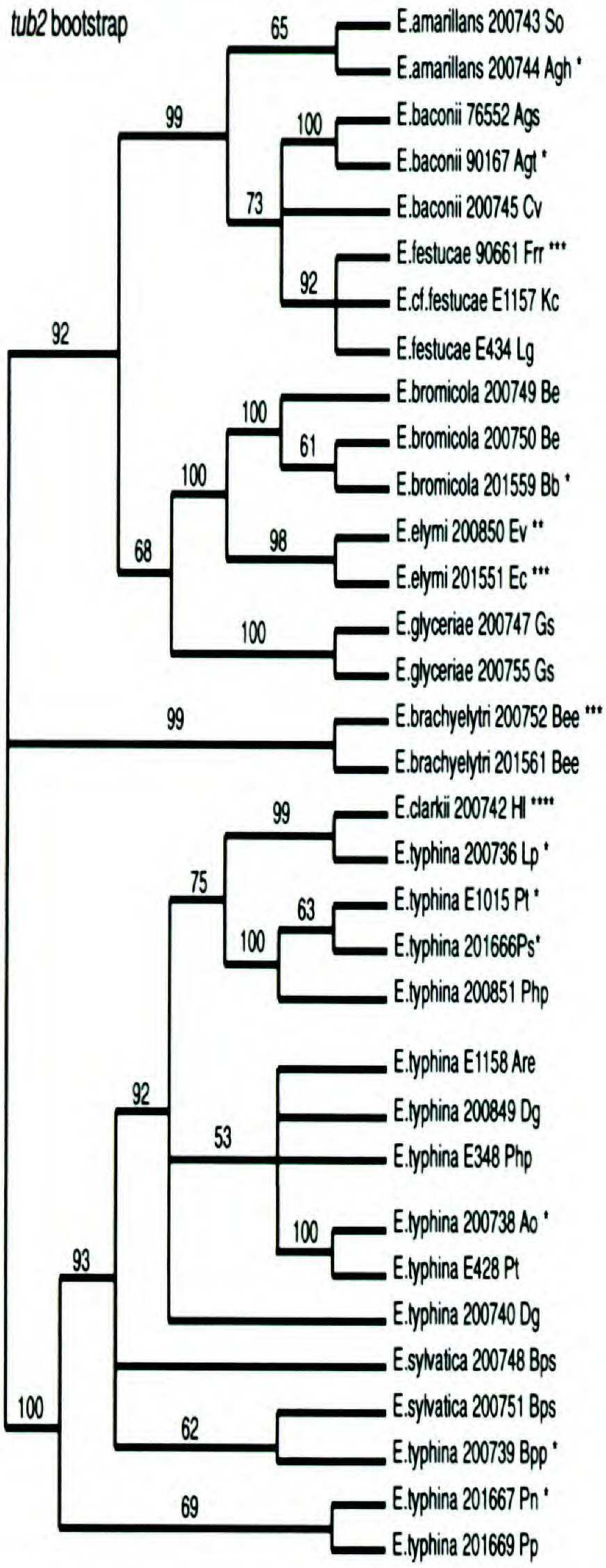


Figure 5. Bootstrap tree generated from *Epichloë tub2* gene sequence using MP (heuristic). Numbers at branches are the percentage of trees containing the corresponding clade based on 1000 bootstrap replications. Bootstrap values greater than 70% are considered supportive of the clades indicated by the branches. For bootstrap analysis each set of identical sequences was represented twice. Asterisks (*) indicate the number of additional isolates (ranging from one to four), sampled from the same host genus, that had identical sequence.

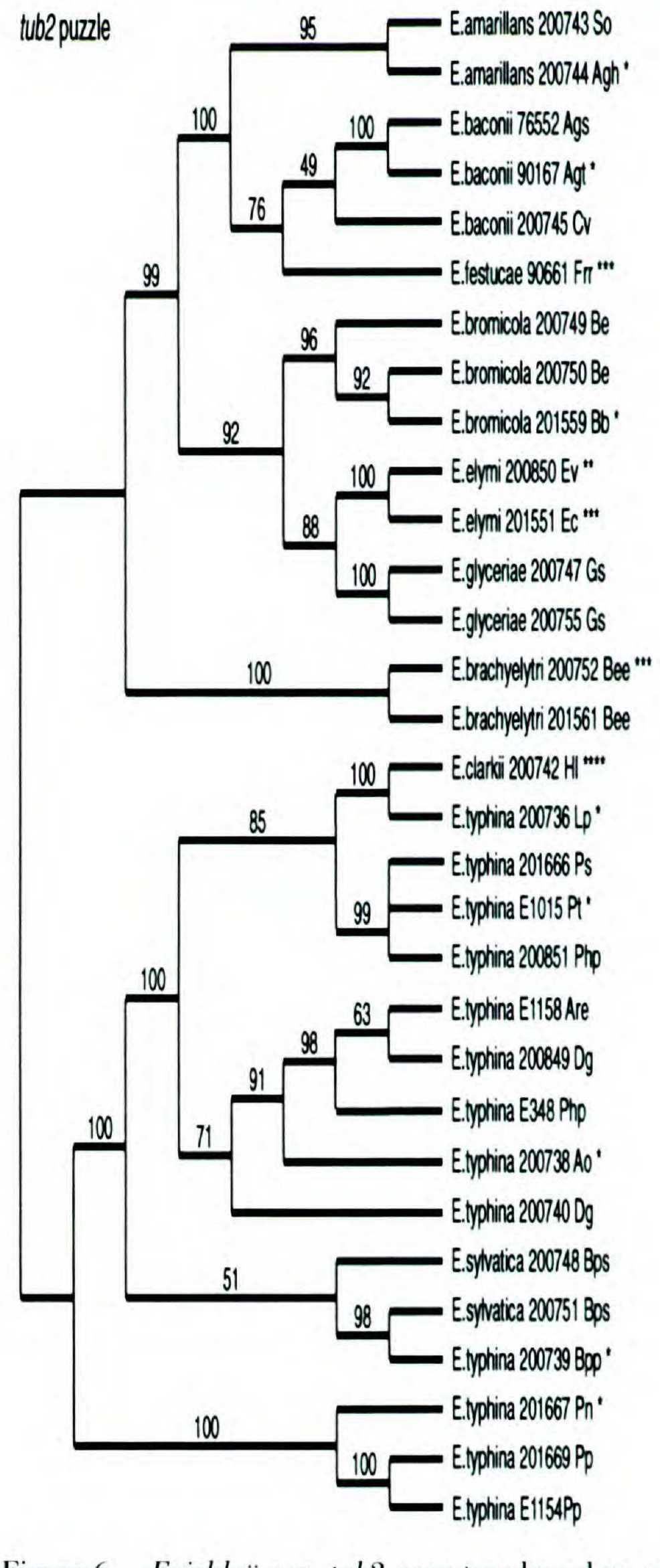


Figure 6. *Epichloë* spp. *tub2* gene tree based on maximum likelihood (ML) PUZZLE analysis of introns 1–3. Tree is based on a Hasegawa-Kishino-Yano model with ts/tv = 2 (kappa = 4.01068); number of puzzling steps = 1000. Tree shown represents majority-rule consensus and numbers at branches are estimations of support for each branch.

Members of most biological species tended to group together. For example, the isolates of *E. amarillans*, *E. brachyelytri*, *E. festucae*, *E. bromicola*, *E. clarkii*, *E. elymi*, and *E. glyceriae* grouped into exclusive clades. However, *E. baconii* from *Agrostis* spp. were not definitively grouped with *E. baconii* from *Calamagrostis villosa*, the two *E. sylvatica* isolates did not definitively group in a monophyletic clade, and *E. typhina* exhibited apparent paraphyly with *E. sylvatica* and *E. clarkii*.

The *tub2* tree included sequences from several new isolates. An isolate from host species *Koeleria cristata* was placed in the *Epichloë festucae* clade, with perfect sequence identity to four other *E. festucae* isolates. New isolates from *Arrhenatherum elatius*, *Poa silvicola*, and *P. trivialis* grouped together with isolates from several other hosts in the crown clade of the *E. typhina* complex. New isolates from *Poa pratensis* and *Brachypodium pin-*

*natum* grouped with other isolates from these same hosts, in basal clades of the *E. typhina* complex.

### TRANSLATION ELONGATION FACTOR 1-α GENE PHYLOGENY

To assess the extent to which the *tub2* gene tree reflected the evolutionary relationships among *Epichloë* species and isolates, noncoding portions of two additional genes, *tef1* and *act1*, were sequenced from most of the isolates. A portion of *tef1* containing introns 1–4 was amplified and sequenced from representatives of the *Epichloë* species, and phylogenetic trees were inferred by MP (Figs. 7, 8), NJ (not shown), and ML quartet puzzling (Fig. 10). The *tef1* MP trees resolved most of the same clades that were resolved in the *tub2* trees, except that the positions of *E. glyceriae* and *E. brachyelytri* were unresolved in the *tef1* trees. Within the *E. typhina* complex *tub2* and *tef1* phylogenies differed in the crown clade, as would be expected if clade members are interbreeding. In bootstrap analysis, all major clades that were strongly supported by the *tub2* data were also strongly supported by *tef1* (Fig. 8). The *tef1* trees grouped isolates of each of the species, *E. brachyelytri*, *E. bromicola*, *E. clarkii*, *E. elymi*, *E. festucae*, and *E. glyceriae*. Furthermore, *E. typhina* isolates from *Lolium perenne*, *Poa nemoralis*, and *Brachypodium pinnatum* grouped into exclusive clades, each with strong bootstrap support.

### ACTIN GENE PHYLOGENY

The *act1* MP trees were not as highly resolved as the other gene trees. This lack of resolution resulted in failure to successfully complete bootstrap analysis on the *act1* data set. Even so, all major clades indicated by *tub2* and *tef1* analyses were supported in the *act1* consensus trees (Fig. 11). One conspicuous incongruence between the *act1* phylogeny and those constructed from both *tub2* and *tef1* was the placement of the isolates within the basal clades of the *Epichloë typhina* complex. Both *tub2* and *tef1* analysis placed the *E. typhina* isolates from *Poa nemoralis* and *P. pratensis* together in the most basal clade, whereas the *act1* trees placed the *E. typhina* isolates from *Brachypodium pinnatum* together with one of the *E. sylvatica* isolates (ATCC 200751) from *Bp. sylvaticum* as the most basal clade (Figs. 11, 12). Such incongruences between gene trees in placement of taxa suggested possible genetic reticulation due to interbreeding within the *E. typhina* complex.

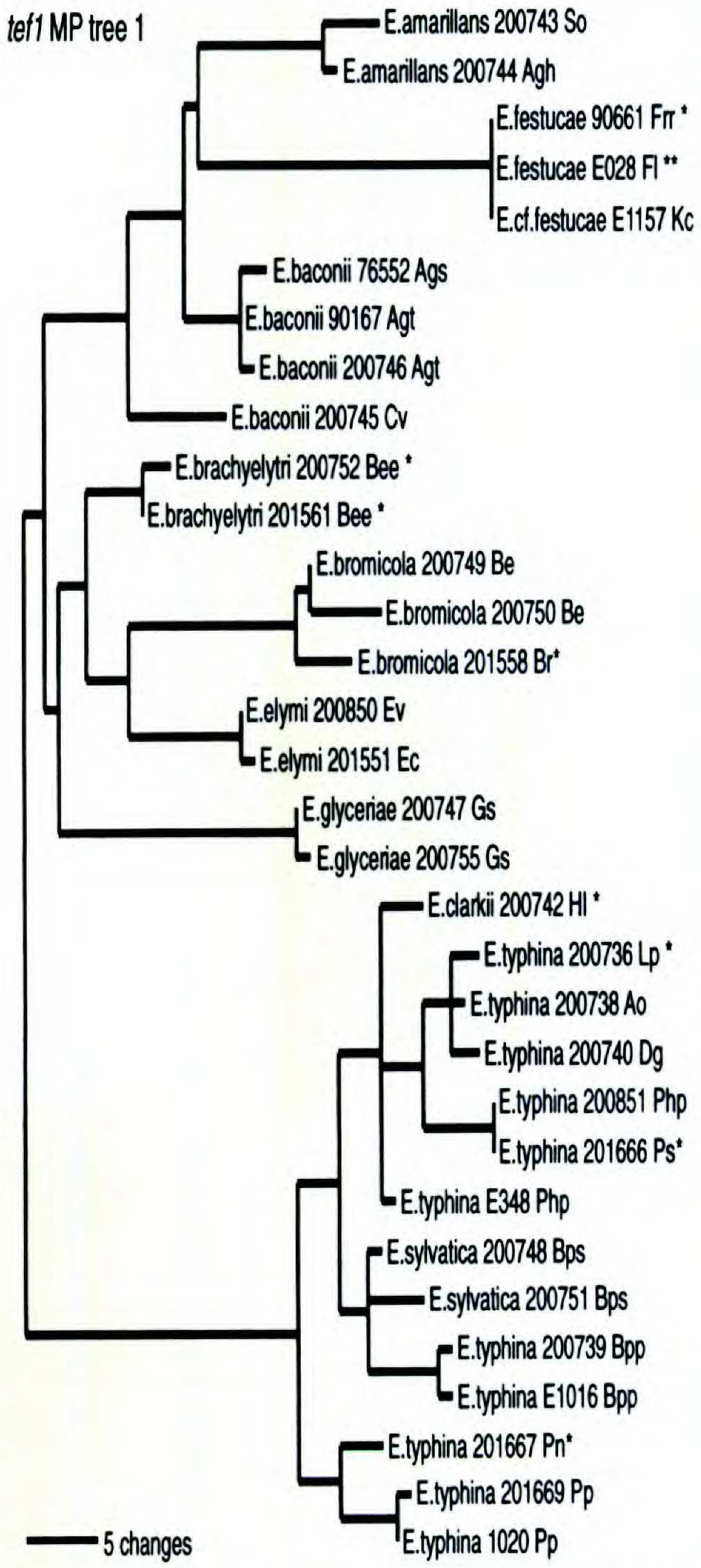


Figure 7. *Epichloë* spp. *tef1* gene tree based on MP analysis of introns 1–4. Branch-and-bound search resulted in 72 MP trees. Number of parsimony-informative characters = 110; tree length = 196 steps; consistency index = 0.8571; retention index = 0.9506; rescaled consistency index = 0.8148; midpoint root is at the left edge. Bar represents five inferred nucleotide substitutions. Asterisks (*) indicate the number of additional isolates (ranging from one to two), sampled from the same host genus, that had identical sequence. Some sequences separated on zero-length branches differ only in indels, which were treated as missing information in this analysis.

### TESTS FOR TREE INCONGRUENCES

To circumscribe interbreeding populations we investigated reticulation within clades. Interbreeding should lead to incongruences between gene trees in the placement of isolates within the interbreeding population. Most clades corresponding to interfertile groups had too little sequence variation to

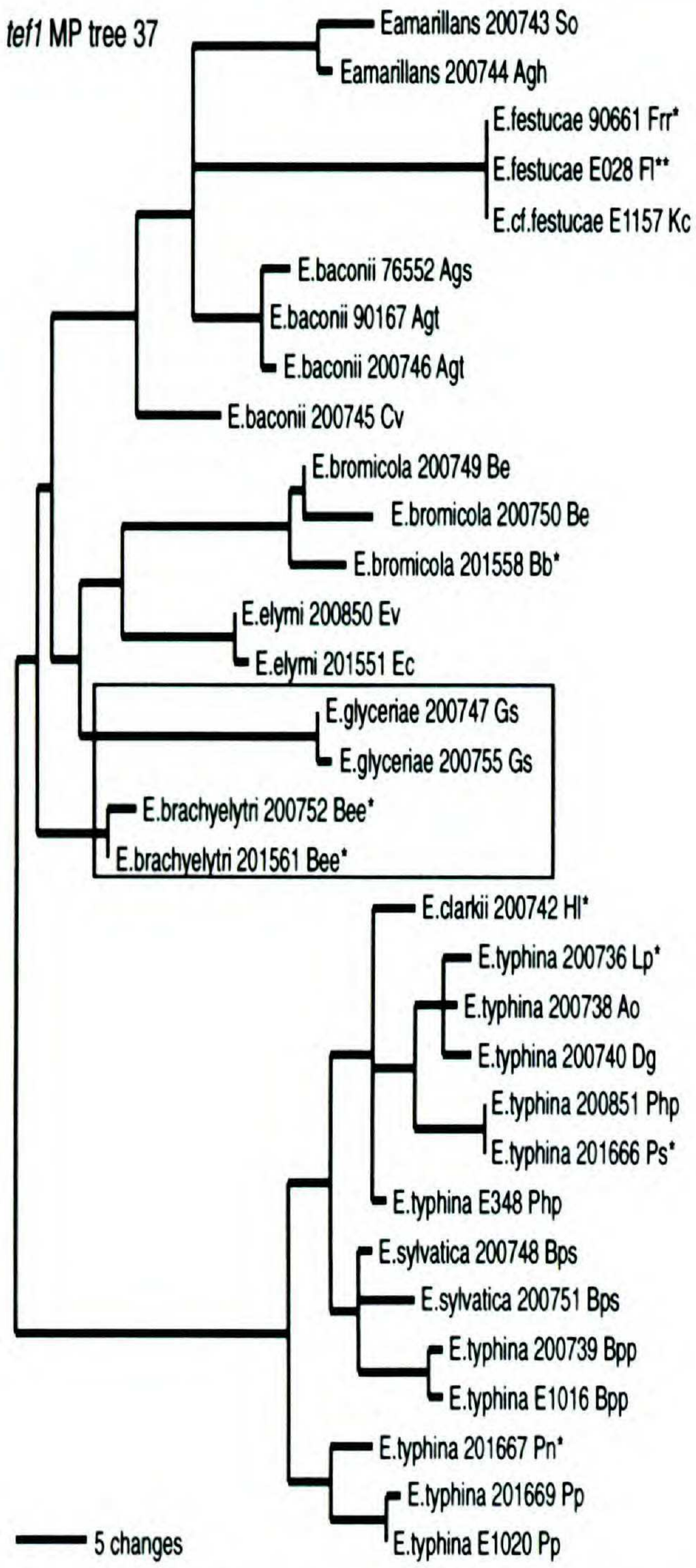


Figure 8. *Epichloë* spp. *tef1* gene tree based on MP analysis of introns 1–4. Branch-and-bound search resulted in 72 MP trees. Tree shown was chosen to represent variation in branching orders among the MP trees. The most important differences are the positions of *E. brachyelytri* and *E. glyceriae* (boxed). Number of parsimony-informative characters = 110; tree length = 196 steps; consistency index = 0.8571; retention index = 0.9506; rescaled consistency index = 0.8148; midpoint root is at the left edge. Bar represents five inferred nucleotide substitutions. Asterisks (*) indicate the number of additional isolates (ranging from one to two), sampled from the same host genus, that had identical sequence. Some sequences separated on zero-length branches differ only in indels, which were treated as missing information in this analysis.

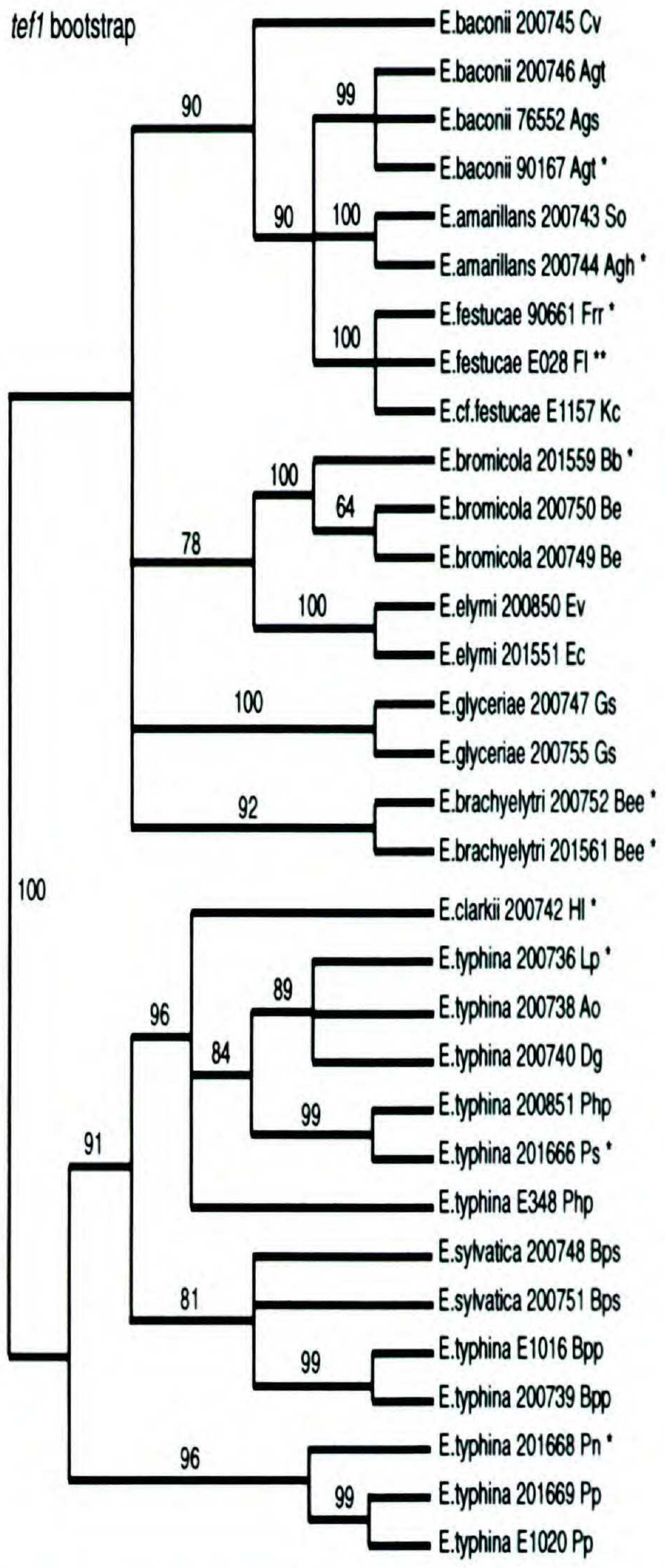


Figure 9. Strict consensus cladogram from the 72 MP trees derived from a branch-and-bound search of *tef1* sequence. Numbers at branches are the percentage of trees containing the corresponding clade based on 500 bootstrap replications. Bootstrap values greater than 70% are considered supportive of the clades indicated by the branches. For bootstrap analysis each set of identical sequences was represented only once.

make such an assessment. However, three such groups were highly diverse in sequences: *Epichloë baconii*, *E. sylvatica*, and the *E. typhina/E. clarkii* group. In all three gene trees (Figs. 4, 7, 11) the *E. baconii* isolate from *Calamagrostis villosa* was well separated from those with *Agrostis* spp. hosts. Thus, there was no conflict between the gene trees that would suggest intermating between *E. baconii* in different clades. There were, however, incongruences between gene trees in the *E. typhina* complex. Specifically, there were differences in the crown clade between all gene trees, and there were differences in basal clades of the complex when the *act1* tree was compared to the *tub2* and *tef1* trees.

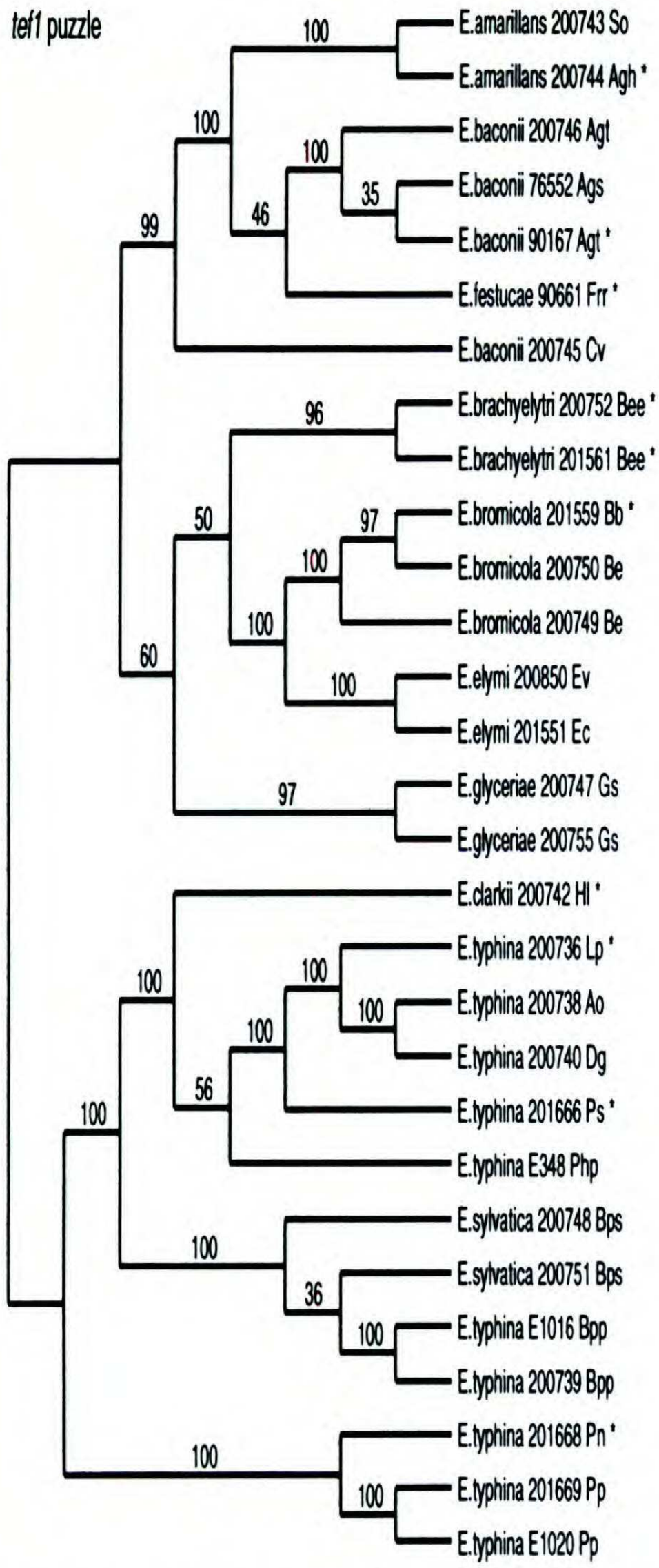


Figure 10. *Epichloë* spp. *tef1* gene tree based on ML puzzle analysis of introns 1–5. Tree is based on a Hasegawa-Kishino-Yano model with ts/tv = 2 (kappa = 3.97409); number of puzzling steps = 1000. Tree shown represents majority-rule consensus, and numbers at branches are estimations of support for each branch.

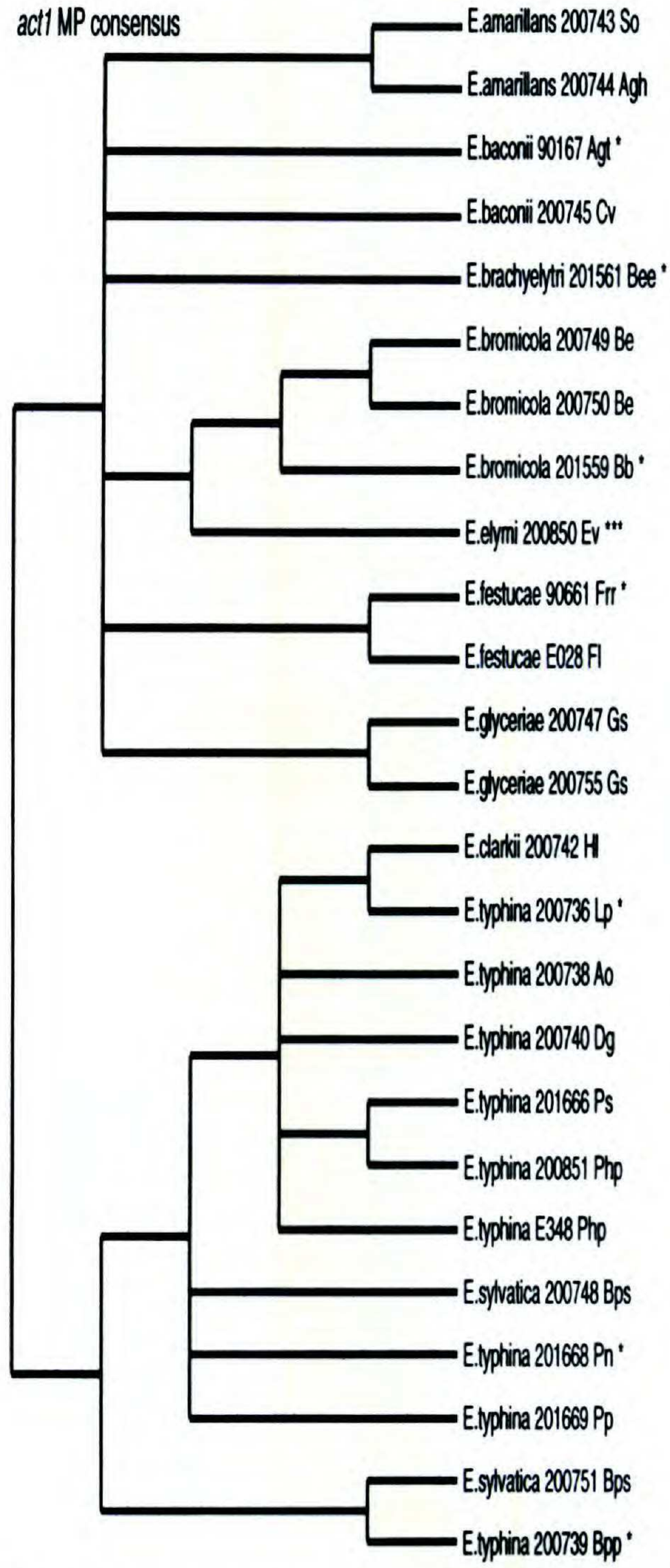


Figure 11. Strict consensus cladogram from the 1946 MP trees derived from a branch-and-bound search of *act1* sequence. Number of parsimony-informative characters = 80; tree length = 149 steps; consistency index = 0.8792; retention index = 0.9430; rescaled consistency index = 0.8291; midpoint root is at the left edge. Asterisks (*) indicate the number of additional isolates (ranging from one to three), sampled from the same host genus, that had identical sequence.

These incongruences and the involvement of these clades were investigated by partition homogeneity tests.

The partition homogeneity test compares phylogenetic information from two genes to determine whether there is statistically significant evidence of incongruence between sequence data sets ($P \leq 0.05$). Since we were interested in reticulation (possible intermating) within the genus *Epichloë*, we removed the outgroup taxa (*C. purpurea* and *Ech. tuberiformis*) from the analysis. Comparison of *act1* with *tef1* resulted in significant incongruence ($P = 0.03$). To determine which taxa may be reticulated, thus the source of incongruence, we removed certain members of the *E. typhina* complex and reanalyzed the data. When all of the crown clade was removed except *E. typhina* from *Dactylis glomerata* and *E. clarkii* from *Holcus lanatus*, and the test was rerun, there was no longer significant incongruence between *act1* and *tef1* trees ($P = 0.20$). Similarly, removal of all *E. typhina* isolates except one from

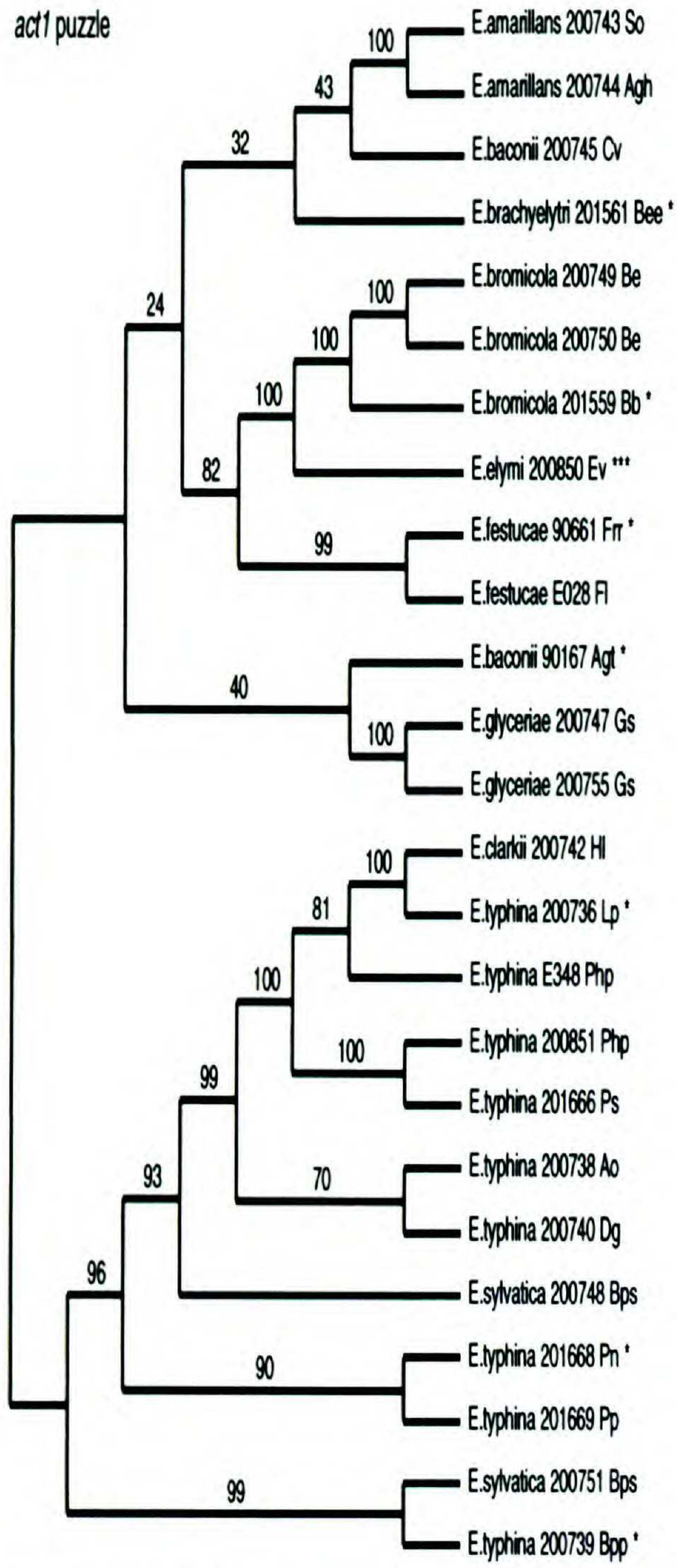


Figure 12. *Epichloë* spp. *act1* gene tree based on ML puzzle analysis of introns 1–4. Tree is based on a Hasegawa-Kishino-Yano model with ts/tv = 2 (kappa = 3.98002); number of puzzling steps = 1000. Tree shown represents majority-rule consensus and numbers at branches are estimations of support for each branch.

*D. glomerata*, while leaving *E. clarkii* and *E. sylvatica*, gave a nonsignificant test result ($P = 0.47$).

Partition homogeneity analysis of *tub2* and *tef1* gave similar results. When the test included all *Epichloë* taxa, there was a significant incongruence between these two gene sets ($P = 0.01$). Removal of all crown clade isolates except *E. typhina* from *Dactylis glomerata* and *E. clarkii* from *Holcus lanatus* resulted in nonsignificance ($P = 0.71$). Elimination of all *E. typhina* isolates except the *D. glomerata* isolate also resulted in nonsignificance ($P = 0.59$).

Inclusion of all *Epichloë* isolates in the *act1/tub2* test indicated incongruence ($P = 0.01$). In contrast to the other gene comparisons, when the test was run without the crown clade *E. typhina* isolates it still indicated incongruence ($P = 0.02$). However, if taxa from *Poa pratensis*, *P. nemoralis*, and *Brachypodium pinnatum* were also pruned from the test, there was no significant incongruence ($P = 0.23$). Thus, evidence of reticulation in this last comparison was only lost once *E. typhina* isolates from both the crown and basal clades were removed, suggesting reticulation throughout the *E. typhina* complex.

## Discussion

### Phylogenetic and Biological Species

The main focus of this study was to examine the relationship between biological and phylogenetic species, as well as the role of ecological specialization in genetic isolation of populations of *Epichloë* species. To address these issues, we sought additional genes to compare to the *tub2* gene trees previously inferred (Schardl et al., 1997). Since genetic linkage tests indicated that *tef1*, *act1*, and *tub2* were not closely linked, at least in *E. typhina*, these were considered appropriate for gene tree comparisons. Taxonomic sampling was comparable in scope to our recent *tub2* phylogenetic analysis (Schardl & Leuchtmann, 1999), with all 10 described species of *Epichloë* sensu stricto represented by multiple isolates. The trees inferred from *tef1* and *act1* intron sequences largely corroborated the relationships within the genus *Epichloë* previously elucidated by *tub2* phylogeny (Tsai et al., 1994; Schardl & Leuchtmann, 1999). Analysis of combined data with outgroups also corroborated, with moderate support, the midpoint root inferred for each of the three gene trees. The tree thus rooted divides the genus into two major clades. One clade comprises most of the currently accepted *Epichloë* species, three of which (*E. baconii*, *E. bromicola*, and *E. festucae*) are associated with Eurasian grass hosts (e.g., *Festuca* spp. and *Bromus* spp.), and four (*E. amarillans*, *E. brachyelytri*, *E. elymi*, and *E. glyceriae*) with North American grasses (e.g., *Elymus* spp. and *Agrostis* spp.). This major *Epichloë* clade we refer to as the main group. The other major clade, here termed the *E. typhina* complex, was associated with a broad range of Eurasian grasses and included the described species *E. typhina*, *E. clarkii*, and *E. sylvatica*. For the most part, the main group of *Epichloë* comprised clear-cut phylogenetic species corresponding to established interfertility

groups and morphologically based species. A possible exception, *E. baconii*, is discussed later, as is the possibility of cryptic biological species within morphospecies *E. festucae* and *E. amarillans*. However, the *E. typhina* complex poses the most difficulty in reconciling phylogenetic and biological species concepts.

Biological species can be defined as potentially interbreeding populations that are reproductively isolated from other mating populations (Claridge et al., 1997; Mayr, 1940). Recent descriptions of *Epichloë* species have considered the results of mating tests to identify potentially interbreeding groups (Leuchtmann et al., 1994; Leuchtmann & Schardl, 1998; Schardl & Leuchtmann, 1999; White, 1993, 1994). However, such tests do not necessarily indicate actual interbreeding populations. This is a particular concern if parents are so specialized to different ecological niches, such as hosts, as to preclude survival of their progeny. A phylogenetic approach to identify effectively interbreeding populations has recently been applied to fungi for which the sexual cycle is unknown (Geiser et al., 1998; Burt et al., 1996). The approach is to identify the smallest well-defined phylogenetic units in combined analysis of multiple genes. Such clades are suggested to indicate reproductively isolated populations (Geiser et al., 1998). One assumption is that phylogenetic species, so defined, are equivalent to biological species. In our study we have looked only at known sexual *Epichloë* species and, furthermore, have identified the interfertility groups in the genus. This allows a check on the relationships between phylogenetic and biological species.

We looked for reticulation that would give rise to disparities between tree topologies inferred from different, unlinked genes. Reticulation indicates that either the population has a history of recombination characteristic of an interbreeding gene pool or that much of the extant polymorphism was inherent in the clade's ancestral gene pool. We point out that this analysis cannot indicate how recombination occurs, how often it occurs, or when it last occurred. Genetic tests indicated that the *tef1*, *act1*, and *tub2* genes independently segregated in *Epichloë typhina*, so we considered these genes appropriate for the analysis. Tests of linkage were most feasible in *E. typhina* because this species had sufficient sequence diversity of all three genes for the appropriate primers to be developed and PCR tests to be conducted. An assumption was that these genes are likewise not closely linked in other *Epichloë* clades. We consider this a reasonable assumption because, since they are not linked in *E. typhina*, it is unlikely that all three would be so closely linked in other clades as to affect inferences about genetic reticulation. More importantly, the most apparent conflicts between phylogenetic and biological species were associated with the *E. typhina* complex.

Likely reticulation was identified by conducting partition homogeneity tests both with all taxa included and with most members of potentially reticulating clades removed. Since all three gene trees were obviously concordant for the main *Epichloë* group, we focused on the *E. typhina* complex, where the different gene trees gave different topologies. Not surprisingly, when all taxa were included the test indicated significant conflict between the trees. In two comparisons (*act1/tef1* and *tub2/tef1*) significance was lost when the *E. typhina* crown clade (including *E. clarkii*) was reduced to two representatives. This result strongly suggests that the crown clade comprises or is a component of a reticulated gene pool. Only when the *E. typhina* complex was further pruned by removal of most basal taxa did the test indicate congruent *act1* and *tub2* trees. This suggests that there was also reticulation in the basal taxa, and inspection of the gene trees indicates that the positions of *E. sylvatica* isolates differ in the different trees. The positions of *E. sylvatica* isolates in the three gene trees were a particular surprise, as was the lack of fertility between *E. sylvatica* and *Brachypodium pinnatum*-associated *E. typhina* despite their close relationships (Leuchtmann & Schardl, 1998). It would appear that *E. sylvatica* has emerged as a distinct species from within *E. typhina*, or that *E. sylvatica* has recombined with *E. typhina* in the course of its evolution prior to, or despite, emergence of intersterility barriers.

It is possible that this conflict between biological and phylogenetic species may be a problem of lineage sorting in the *Epichloë typhina* complex. Lineage sorting arises due to polymorphisms present in an ancestral mating population from which different alleles are fixed in extant lineages, and can result in incorrect phylogenetic inferences being drawn as to the true relationships between extant lineages (Lyons-Weiler & Milinkovitch, 1997). Another possibility is that there has been parasexual recombination involving *E. typhina* and *E. sylvatica* (Caten, 1981). This is plausible considering the origins of many asexual endophytes as hybrids of multiple *Epichloë* species. However, the hybrid asexual species generally have large portions of the genomes of their ancestors, thus containing multiple copies of most genes (Kuldau et al., 1999). There is no indication of this in *Epichloë* species from analysis of *tub2*, *tef1*, and *act1*, though occa-

sional multiband phenotypes have been observed in isozyme studies of *E. typhina* (Leuchtmann & Schardl, 1998). Even if parasexual recombination occurs in the *E. typhina* complex, the close relationship between *E. sylvatica* ATCC 200751 and *E. typhina* from *Brachypodium pinnatum* is unexpected in that all three gene trees give this same relationship, and isozyme analysis gives a similar result (Leuchtmann & Schardl, 1998). In stark contrast, *E. typhina* from *Bp. pinnatum* did not share any identical or highly similar gene sequences with *E. typhina* isolates from any other host, as would have been expected from either sexual or parasexual recombination. Although we cannot exclude either lineage sorting or parasexual recombination to explain the apparent paraphyly of *E. typhina* to *E. sylvatica*, as well as polyphyly of both biological species, these relationships nevertheless amount to a conflict between the phylogenetic and biological species concepts.

With the exception of the *E. typhina* complex, most phylogenetic species of *Epichloë* had little genetic diversity, and there is little or no evidence of gene flow (reticulation) between phylogenetic species. Previous mating test results (Schardl & Leuchtmann, 1999) have shown that many phylogenetic species in the main *Epichloë* group also conform to a biological species concept; there was intermating only between isolates from the same or closely related hosts, often within one host genus. Yet, even in the main group there were some cases where phylogenetic species did not precisely predict interfertility groups or vice versa. *Epichloë baconii* isolates from host genera *Agrostis* and *Calamagrostis*, though capable of intermating, never definitively grouped in a monophyletic clade in any gene phylogram (this was also the observation based on allozyme cluster analysis; Leuchtmann & Schardl, 1998). However, it is possible that whenever *Agrostis*- and *Calamagrostis*-associated populations intermate in nature their progeny are lost due to lack of a compatible host. Such a situation would isolate the populations into different biological species despite their observed interfertility in experimental matings. More sampling of the *E. baconii* populations is needed to assess if they actually represent distinct biological species or, alternatively, if this is another conflict between phylogenetic and biological species concepts.

The converse situation apparently arises with *Epichloë amarillans* and *E. festucae*. An *E. amarillans* isolate on *Sphenopholis obtusata* had limited or no interfertility with an isolate from *Agrostis hiemalis*. Although mating proceeded as expected, progeny showed no evidence of genetic contribution from the male. Though this does not preclude limited genetic exchange, the result suggests that these host-associated populations have a degree of genetic isolation. Since phylogenetic analysis placed these populations into distinct clades they may indeed represent different biological species. Likewise, *E. festucae* from *Festuca rubra* failed to mate with an isolate from *Koeleria cristata* even though they had identical gene sequences. This test must be considered preliminary pending reciprocal mating tests. Nevertheless, the result points up a potential limitation that if sequence evolution does not adequately keep pace with biological speciation it may be difficult to identify species on a strictly phylogenetic basis.

### GENE FLOW WITHIN A BIOLOGICAL SPECIES

The relationships among host-associated populations in the *Epichloë typhina* complex is intriguing. In keeping with previous results from isozyme analysis (Leuchtmann & Schardl, 1998) we found that certain host grasses harbor genetically diverse endophyte populations, whereas other hosts harbor populations of genetically similar individuals. Among the more diverse are the *E. typhina* populations in *Dactylis glomerata* and *Phleum pratense*, as well as *E. sylvatica* from *Brachypodium sylvaticum*. In contrast, isolates from other host-associated fungal populations had similar or identical allozyme profiles and DNA sequences. Little or no sequence variation was observed within *E. typhina* populations in many of its grass hosts: *Anthoxanthum odoratum* (based on *tub2*), *Brachypodium pinnatum* (*tub2* and *tef1*), *Lolium perenne* (*tub2*, *tef1*, and *act1*), *Poa nemoralis* (*tub2*, *tef1*, and *act1*), and *P. pratensis* (*tub2*); as well as among *E. clarkii* from *Holcus lanatus* (*tub2* and *tef1*). Since most of these host-associated populations are currently represented by a small sample size it is unclear whether they might be substantially more diverse than so far indicated. Still, the genetic uniformity of *E. clarkii* seems well substantiated because this species is represented by five isolates from Britain and Switzerland, and both mating types are included. Even though most *E. clarkii* isolates are readily interfertile with *E. typhina* on several other hosts, *E. clarkii* seems to maintain genetic and even morphological distinction from the rest of the *E. typhina* complex (White, 1993; Leuchtmann & Schardl, 1998). However, the position of *E. clarkii* relative to the various *E. typhina* isolates is not consistent among gene trees, so that *E. clarkii* does not fit the criterion for a phylogenetic species. It is possible that *E. typhina* on other grasses—such as *Dactylis*

*glomerata*—actually mates with *E. clarkii* in nature, but that the progeny do not colonize or survive on *Holcus lanatus*, host grass for *E. clarkii*. Perhaps only *E. clarkii* × *E. clarkii* progeny can colonize this host, leading to extreme inbreeding but not necessarily genetic isolation. Thus, *E. clarkii* may contribute to the gene pool of certain *E. typhina* populations, but *E. typhina* rarely or never contributes to the *E. clarkii* (i.e., *H. lanatus*-associated) gene pool. The other low-diversity host-associated populations may have similar relationships to the more diverse ones.

Is incipient speciation occurring in the *Epichloë typhina* complex? One might expect speciation based on host-associated populations given the strong association of other *Epichloë* species with individual host genera or tribes. *Epichloe sylvatica* appears to be an example, since it is restricted to one host, *Brachypodium sylvaticum*. Yet even if such species emerge, it appears that they will not correspond to such clear-cut phylogenetic clades as do the other *Epichloë* species. This, also, is illustrated by the apparent polyphyly of *E. sylvatica*. Overall, no phylogenetic species obviously emerge from host-associated populations in the *E. typhina* complex. However, it appears that the crown clade of *E. typhina/E. clarkii* may have effectively speciated from the more basal clades. All indications of reticulation were either within the crown clade or among the basal clades, but not between the crown and basal clades. This suggests that the crown clade is an incipient species that has not fully established genetic barriers to mating outside the clade (many matings between basal and crown clade members are fertile; Leuchtmann & Schardl, 1998). Furthermore, even though one genus (*Poa*) is represented among hosts of both basal and crown clades, the different clades consistently comprise isolates from different host species. *Epichloë* species in the crown clade infect *P. silvicola* and *P. trivialis*, whereas members of the more basal clades infect *P. pratensis* and *P. nemoralis*.

## PREVAILING TRANSMISSION MODES AND EVOLUTION

The apparently different evolutionary processes between the *Epichloë typhina* complex and the "main group" containing the other seven *Epichloë* species may relate to their different host interactions, namely whether they are more benign and vertically transmissible or more virulent (antagonistic) and reliant on horizontal transmission. Most members of the *E. typhina* complex are antagonistic (strong chokers) in that they shut down almost all host seed production. They must therefore rely on horizontal transmission. As discussed above, speciation in this complex is not clear cut. In contrast, most of the species in the main group balance vertical and horizontal transmission, and, as partial chokers, have more benign or even mutualistic effects on host plants. In this group speciation follows a cladistic stereotype and most species boundaries are unambiguous.

Species in the main *Epichloë* group with substantial seed transmission include *E. brachyelytri*, *E. amarillans*, *E. festucae*, and *E. elymi* (Leuchtmann & Schardl, 1998; Leuchtmann et al., 1994; Schardl & Leuchtmann, 1999). The situation for *E. bromicola* is unique in that it is a strong choker on *Bromus erectus*, but is strictly seed transmitted on other *Bromus* species (Leuchtmann & Schardl, 1998). It is possible that *E. bromicola* has both transmission modes on an unidentified host. Only two main group species, *E. glyceriae* and *E. baconii*, are strong chokers for which vertical transmission is unknown. The deep rooting of *E. brachyelytri* (based on combined sequence analysis; Fig. 3) and the preponderance of vertically transmitted species suggest that this is an ancestral trait of the main *Epichloë* group. We previously proposed that the loss of seed transmissibility in *E. glyceriae* might have been associated with a host jump, since without this fungal species the relationships among the main *Epichloë* group largely reflect host relationships (Schardl et al., 1997). However, since the relevant branches were unresolved in *tef1* and *act1* trees, it remains possible that *E. glyceriae* and the rest of the main group have tracked the evolutionary paths of their hosts.

Even with inconsistent phylogenetic tracking it is likely that host specialization played a role in *Epichloë* speciation. Since vertical transmission provides a dependable route for dissemination there is little benefit expected for broadening host range. This is particularly true if there is a tradeoff between broad host range and the balance of vertical and horizontal transmission in these fungi. We have suggested that such a balance of host and fungal development is unlikely to survive host jumps, and that this is why the more mutualistic *Epichloë* species seem to track host phylogeny (Schardl et al., 1997). Here we suggest that this is also why there are clear boundaries between host-associated species in the main *Epichloë* group. As further evidence that benign or mutualistic interactions have promoted host-based speciation, the phylogenetic pattern is substantially different for the more antagonistic species, especially the *E. typhina* complex. Models of host-parasite interactions predict that high virulence levels (antagonism) should cor-

relate with rapid contagious spread (Yamamura, 1993). No comparative study has been done on transmission rates of *Epichloë* species. However, horizontal transmission of *E. typhina* appears to be very rapid in *Dactylis glomerata* populations, as indicated by a recent epidemic in Oregon (Pfender & Alderman, 1999). We speculate that high virulence and reliance on horizontal transmission selects for broad host range and limits speciation in the *E. typhina* complex. There are two reasons why *E. typhina* may be particularly subject to this kind of selection. First, since this species depends heavily on horizontal transmission the availability of hosts within a short range of a parent stroma (specifically, the dispersal area for ascospores) is important for its propagation. Second, it can participate in reducing the host population, and indeed it will be a factor in the reduction of available hosts by reducing flowering. Whenever local host populations decline either by effects of *E. typhina* or other factors, there would be increased selection on the fungus to colonize other hosts. Such a selective effect is not expected for more benign *Epichloë* species because their assured vertical dissemination obviates any immediate reliance on horizontal transmission to neighboring plants.

## ORIGINS OF THE MUTUALISTIC ENDOPHYTES

The main group of *Epichloë* species represents the emergence of a mechanism to maximize mutual benefit to host and symbiont. This mechanism is a balanced interaction whereby individual plant-fungus symbiota can simultaneously exhibit sexual propagation of both partners. Although the necessary components of such interactions are represented among related Clavicipitaceae (Clay & Jones, 1984; Kover & Clay, 1998), it is only in the main *Epichloë* group that such a balance is consistently evident (Schardl & Clay, 1997). In contrast, the more antagonistic effects of the *E. typhina* complex are similar to those of many other plant-associated Clavicipitaceae, particularly *Balansia* species (Diehl, 1950; Kuldau et al., 1997). Furthermore, most of the *E. typhina* complex, like most *Balansia* species, fail to transmit in host seeds. Although *E. typhina* on *Poa nemoralis* can transmit vertically, it tends to choke the vast majority of tillers and thereby severely limit both host seed production and its potential for vertical transmission. Likewise, although *E. sylvatica* was previously considered to have balanced host interactions, more detailed examination has indicated that stroma-forming genotypes are actually strong chokers. What were previously thought to be balanced interactions were simultaneous infections, whereby individual *Brachypodium sylvaticum* plants had two endophytes, one sexual (*E. sylvatica*) and the other asexual (Meijer & Leuchtmann, 1999). Though closely related to *E. sylvatica*, these asexual endophytes represent the opposite extreme in that they have sacrificed all capacity for horizontal spread and are strictly vertically transmitted. Although this may well benefit the host, it is nevertheless apparent that the balanced interactions characterizing much of the *Epichloë* main group species are absent in the *E. typhina* complex as they are among other Clavicipitaceae.

Symbioses of asexual endophytes with grasses represent a third class of fungal/host interactions distinct from the balanced or antagonistic associations involving sexual *Epichloë* species. Many grasses in subfamily Pooideae possess asexual derivatives of *Epichloë* species, which, by current taxonomic convention, are classified in a distinct genus in the Fungi Imperfecti. Several such species have been formally described and placed in genus *Neotyphodium* (Glenn et al., 1996). These endophytes are often beneficial to their hosts and cause no disease, so they are commonly regarded as mutualists. We are currently analyzing *tub2*, *tef1*, and *act1* noncoding regions for several *Neotyphodium* species, some formally described and others remaining to be described. Results from this *tub2* analysis indicate that several have hybrid origin (Schardl et al., 1994; Tsai et al., 1994; Moon et al., 2000), a conclusion also supported by analysis of *tef1* and *act1* genes (K.D.C. & C.L.S., unpublished obs.). As we expand the number of genes and species analyzed it is becoming apparent that hybrid origins are very common among *Neotyphodium* spp., though not universal. For example, the asexual *N. coenophialum*, a common endophyte of *Lolium arundinaceum* (= *Festuca arundinacea*), has three *Epichloë* species in its pedigree (Tsai et al., 1994); whereas *N. lolii*, the most common endophyte of *Lolium perenne*, seems to be a direct descendant of *E. festucae*. Those *Epichloë* species involved in known hybridizations span the phylogenetic diversity of the genus, including *E. typhina*, *E. bromicola*, *E. elymi*, *E. amarillans*, *E. festucae*, and a relative of *E. baconii*. The hybridizations have led to diploidy, polyploidy, or extensive aneuploidy in most *Neotyphodium* species studied to date, and for this and other reasons are likely somatic events rather than hybrid matings (Schardl et al., 1994; Kuldau et al., 1999). Thus, traditional species concepts are violated, albeit in different ways, both by endophytes that only trans-

mit vertically and those that only transmit horizontally.

## Conclusions

The most striking result from our phylogenetic studies of *Epichloë* species and their asexual relatives is that different evolutionary patterns are associated with different modes of transmission to new host plants. A cladistic pattern, usually assumed to describe speciation in most organisms, was only apparent for one of the two major lineages in the *Epichloë* phylogeny. Surprisingly, this was the group that had an exquisite balance of horizontal and vertical transmission uncharacteristic of the fungal family and, indeed, uncharacteristic of most pathogens and symbionts. In contrast, cladistic evolution is less evident both for strictly vertically transmitted and strictly horizontally transmitted species. Interspecific hybridization is often evident in the origins of the vertically transmitted, asexual symbionts (Schardl et al., 1994; Tsai et al., 1994). Further, the *E. typhina* complex—dominated by strains that only transmit horizontally—lacks clear-cut genetically isolated groups (this study). We speculate that high virulence and dependence on horizontal transmission might counteract host specialization and, in turn, promote intraspecies diversity and delay speciation. Delayed speciation should amplify lineage sorting problems that cause conflict between the biological and phylogenetic species concepts, as discussed above. If a reliance on horizontal transmission delays speciation as we speculate, an obvious prediction is that other highly virulent pathogens will exhibit conflicts between biological and phylogenetic species. To our knowledge, this has not yet been tested. A good relationship between these species concepts was noted for the *Gibberella fujikuroi* group (O'Donnell et al., 1998), but these fungi also exhibit both horizontal and vertical transmission (Bacon & Hinton, 1996). Detailed phylogenies and population surveys are clearly needed for more virulent pathogens, organisms that cause the most problems in human health and the food supply.

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